DECLARATION

Mizoram University

June, 2021

I Loknath Samanta, hereby declare that the subject matter of this thesis is the record of work done by me, that the content of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Biotechnology.

(Candidate)

(Head of Department) (Supervisor)

CERTIFICATE

I certify that the thesis entitled "**Studies on physiological and biochemical responses of *Nostoc punctiforme* ATCC 29133 and *Anabaena* sp. PCC 7120 to oxidative stress induced by peroxides"** submitted to the Mizoram University for the award of a degree of Doctor of Philosophy in Biotechnology by Loknath Samanta is a record of research work carried out by him during the period from 2014 to 2021 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles in this University or any other University or institution of higher learning.

Signature of the Supervisor

(Dr. Jyotirmoy Bhattacharya)

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ABBREVIATIONS

°C	Degree Celsius
$CaCl_2.2H_2O$	Calcium chloride dihydrate
$Co(NO_3)_2.6H_2O$,	Cobalt (II) nitrate hexahydrate
$CuSO_4.5H_2O$	Copper sulphate pentahydrate
EDTA	Ethylenediaminetetraacetic acid
H_2O_2	Hydrogen peroxide
H_3BO_3	Boric Acid
K_2HPO_4	Dipotassium phosphate
M	Molar
min	Minute(s)
ml	Millilitre
mM	Millimolar
$MgCl_2$	Magnesium chloride
$MnCl_2.4H_2O$	Manganese chloride tetrahydrate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
MV	Methyl Viologen
Na_2CO_3	Sodium carbonate (anhydrous)
NaCl	Sodium chloride
$Na_2MoO_4.2H_2O$	Sodium molybdate
N_2	Dinitrogen
PMSF	Phenylmethylsulfonyl fluoride

OD	Optical Density
sec	second(s)
Tris (free base)	2-amino-2-hydroxymethyl propane-1,3-diol
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate
%	Percent
µg	Microgram
µmol	Micromole
µM	Micromolar

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STUDIES ON PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES *OF NOSTOC PUNCTIFORME* ATCC 29133 AND *ANABAENA* SP. PCC 7120 TO OXIDATIVE STRESS INDUCED BY PEROXIDES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

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CHAPTER 1

Introduction

1.1 Cyanobacteria

Cyanobacteria, previously known as blue-green algae, are Gram-negative prokaryotes, which originated on Earth some 3.2 billion years ago. They are widely accepted as the first organisms to have started oxygen evolution on Earth through their photosynthetic activity (Dietrich et al. 2006). It is largely because of them that the erstwhile anaerobic atmosphere of Earth gradually transformed into the one that is aerobic today. This oxidation event is believed to be at the core of evolution of aerobic lifestyle on our planet. The present-day chloroplasts of higher plants also owe their origin to cyanobacteria. Not surprisingly therefore, the photosynthetic machinery of cyanobacteria and higher plants are very similar to each other (Whitton and Potts 2000). In cyanobacteria, photosynthesis is driven by the light harvesting phycobilisomes located on the thylakoid membranes. The phycobilisomes consist of a core made up of the pigment allophycocyanin and rods made up of the pigments phycoerythrin and phycocyanin (MacColl 1998). The phycobilisomes transfer light energy to the photosystems I and II (PSI and PSII). Oxidation of water occurs in PSII pigment-protein complex consisting of reaction center proteins D1 and D2 and several redox cofactors that participate in transporting electrons to PSI (Ferreira et al. 2004). PSI is a multi-subunit pigment-protein complex, which generates reduced ferredoxin from PSII-donated electrons (linear electron flow). The reduced ferredoxin then donates electrons to $NADP^+$ (oxidized nicotinamide adenine dinucleotide phosphate) to form NADPH, and also drives cyclic electron flow

around PSI forming ATP. As in higher plants, NADPH and ATP fuel fixation of carbon dioxide into carbohydrates (Calvin cycle) (Golbeck 1992).

Since their advent, cyanobacteria have evolved as one of the most diverse class of microorganisms. They exhibit different morphologies, including unicellular, filamentous, branched-filamentous and non-filamentous colonial forms (Whitton and Potts 2000). These photoautotrophs have also marked their presence in almost every ecological habitat on Earth ranging from fresh water bodies to oceans, polar arctic regions to deserts, and from nutrient-rich soils to nutrient poor usar soils. While many of them are free-living, some are symbiotic associates of plants and other eukaryotes (Adams 2000; Rai et al. 2000). Another worth mentioning characteristic of many cyanobacteria is their ability to fix atmospheric dinitrogen (N_2). Among such cyanobacteria, the filamentous forms, particularly the species of *Nostoc* and *Anabaena*, perform N_2-fixation in specialized microaerobic heterocysts (5-10 % of cells in a filament), formed from vegetative cells (sites for O_2-evolving photosynthetic activity) in response to deficiency of fixed N-sources, such as ammonium, nitrate, and amino acids (Muro-Pastor et al. 2005; Frias and Flores 2015). The ability to photosynthesize and fix N_2 is critical to their role in maintaining carbon and nitrogen cycling on Earth. Besides being ecologically important, they are also being viewed as a sustainable source of environment-friendly biofertilizers (alternative to chemical nitrogen fertilizers), bio-factories for production of many useful bio-chemicals, and bio-fuels (Singh et al. 2016; Kitchener and Grunden 2018).

Relevant to ecological, agricultural, and biotechnological potential of cyanobacteria is their ability to tolerate different environmental stresses (Kitchener and Grunden 2018). Previous studies show that cyanobacteria can adapt to various abiotic

stresses, such as fluctuations in light intensity, nutrient availability, high salinity, drought, temperature, ultraviolet rays, herbicides and heavy metals, among many others (Latifi et al. 2009; Chauvat and Chauvat 2015). Although each of these stressors evokes specific adaptive responses in cyanobacteria, most of them end up enhancing the levels of partially reduced oxygen intermediates, also commonly known as reactive oxygen species (ROS), within cells (Mironov et al. 2019). The ROS commonly encountered by photosynthetic cyanobacteria are singlet oxygen ($^1O_2$), superoxide anion radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\bullet}$) (Latifi et al. 2009; Banerjee et al. 2013). Such ROS trigger oxidative stress in cyanobacteria, a physiological condition, which is potentially destructive to cellular proteins, nucleic acids, and lipids. Reported evidence indicates that severe oxidative stress can lead to loss of membrane integrity, photosynthetic activity, nitrogenase activity, and many other vital metabolic activities in cyanobacteria culminating in death (Latifi et al. 2009). Thus, the ability to tolerate oxidative stress may be one of the critical factors in determining growth and survival of cyanobacteria in their natural habitats, and may also influence their potential for biotechnological applications.

1.2 Antioxidative defence mechanisms in cyanobacteria:

Cyanobacteria have evolved multiple strategies to detoxify ROS. They are probably one of the first organisms to have evolved an elaborate ROS scavenging system, because of their long history of association with oxygen. The ROS scavenging mechanisms in cyanobacteria include non-enzymatic antioxidants such as glutathione,

tocopherols, carotenoids etc., and multiple antioxidative enzymes such as superoxide dismutase, catalase, and peroxidase (Latifi et al. 2009; Banerjee et al. 2013).

1.2.1 Nonenzymatic antioxidants:

The nonenzymatic thiol molecule glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) abundant in cells of cyanobacteria (1-10 mM). This tripeptide is synthesized by sequential action of two enzymes; Glu-Cys Ligase and glutathione synthetase. A cell protective role for this tripeptide alone or in combination with glutathione-S-transferase (GST) has been proposed during oxidative stress induced by heavy metals, organic hydroperoxides and many other oxidants (Kammersheit et al. 2019). Some studies also suggest that GSH may also have a role in protection of PSII in cyanobacteria. Earlier studies reveal that α-tocopherol (vitamin E), a lipid-soluble organic molecule, is also involved in oxidative stress tolerance, and prevent damage to membrane lipids against peroxidation in high light and polyunsaturated fatty acids treated *Synechocystis* sp. PCC 6803 (Maeda et al. 2005). The carotenoid pigments, such as myxoxanthophyll, β-carotene, zeaxanthin, and echinenone have also been shown to reduce oxidative stress in cyanobacteria by dissipating excess light energy from photosensitized chlorophyll, and prevent toxicity imposed by $^1O_2$, an unusual ROS generated as a result of excitation of oxygen ($^3O_2$, ground triplet state) by the reaction center chlorophyll when in triplet excited state during photosynthesis (Latifi et al. 2009).

1.2.2 $O_2^{\cdot-}$ production, toxicity and detoxification mechanisms:

$O_2^{\cdot-}$ is one of the most common ROS produced in photosynthetic cyanobacteria and other aerobes. This ROS is generated by univalent reduction of oxygen by electrons leaked from the photosynthetic electron transport chain (reducing side of PSI and the acceptor side of PSII). $O_2^{\cdot-}$ has a short half-life (2-4 microseconds). As it is negatively charged, it cannot traverse across membranes. Despite this, when produced in excess, such as in the presence of the herbicide methyl viologen or other oxidative stress generating conditions, $O_2^{\cdot-}$ can adversely affect many cellular metabolic pathways by damaging iron-sulphur proteins leading to the release of ferrous iron, which can further generate other ROS (Banerjee et al. 2013). Thus, cells must eliminate $O_2^{\cdot-}$ promptly to prevent death.

Superoxide dismutase (SOD) is the primary enzyme for decomposition of $O_2^{\cdot-}$ in cyanobacteria. SOD decomposes $O_2^{\cdot-}$ into H_2O_2 and O_2 ($2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$). Based on their metal cofactors, SODs are classified into four types: iron- (FeSOD/SodB), manganese- (MnSOD/SodA), copper and zinc- (Cu-ZnSOD/SodC), and nickel-dependent (NiSOD/SodN). All four types of SODs have been detected in cyanobacteria. While most cyanobacterial species contain Fe and MnSOD, Cu/Zn and NiSOD are rare and present in only few marine species. For example, a high-light inducible Cu/Zn SOD has been detected in marine *Synechococcus* sp. WH 7803, and NiSOD in species of *Prochlorococcus* (Latifi et al. 2009; Banerjee et al. 2013).

The freshwater unicellular non-N_2-fixing cyanobacteria, such as *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942 etc., has been shown to possess a single FeSOD (Kim and Suh 2005). MnSOD has not been detected in such cyanobacteria. On the other hand, the filamentous heterocystous N_2-fixing cyanobacteria *Anabaena* PCC 7120,

Anabaena variabilis etc. possess a single Fe and a MnSOD (Banerjee et al. 2013). The presence of multiple MnSODs and a single FeSOD has also been noted in some filamentous cyanobacteria like *Plectonema boryanum* and *Nostoc punctiforme* ATCC 29133 (Campbell and Laudenbach 1995; Moirangthem et al. 2014). The biochemical properties of cyanobacterial FeSOD have been characterized in details revealing that the protein is a dimer sensitive to its own product H_2O_2. The molecular weight of FeSOD monomer derived from various unicellular and filamentous cyanobacteria ranges from 21-25 kDa (Ismaiel et al. 2014). Localization studies have revealed that FeSOD is a cytosolic protein and, in filamentous cyanobacteria it is present in both vegetative cells and heterocysts (Regelsberger et al. 2004). Functional studies indicate that FeSOD is light inducible in *Synechocystis* sp. PCC 6803 (Kim and Suh 2005). In *Synechococcus* sp. PCC 7942, FeSOD has been implicated in tolerance to oxidative stress induced by methyl viologen and low temperature stress (Thomas et al. 1998). However, studies on filamentous cyanobacteria (*Plectonema boryanum*, *Anabaena* PCC 7120, *Nostoc punctiforme* ATCC 29133) have shown that the expression of FeSOD decreases during methyl viologen stress (Campbell and Laudenbach 1995; Raghavan et al. 2011; Moirangthem et al. 2014). Interestingly, overexpression of FeSOD was found to be toxic to growth of *Anabaena* PCC 7120 in the absence of nitrate (N_2-fixing growth) and, overexpressed FeSOD was found to prevent MV-induced death only when this cyanobacterium was grown in the presence of nitrate (Raghavan et al. 2011). This may suggest that the protective effect of FeSOD is nitrogen source dependent in *Anabaena* PCC 7120, a hypothesis which remains to be verified in other filamentous cyanobacteria.

As stated earlier, in addition to FeSOD, most filamentous cyanobacteria also possess one/multiple MnSODs. The MnSOD present in *Anabaena* PCC 7120 has been well characterized. Like FeSOD, MnSOD is also a homodimer. In *Anabaena* PCC 7120, MnSOD is a 30 kDa protein present in thylakoid membranes in both vegetative cells and heterocysts (Regelsberger et al. 2002). This protein has been shown to be essential in preventing damage to PSI, PSII and nitrogenase during oxidative stress in *Anabaena* PCC 7120. Further, overexpression studies have revealed that MnSOD confers protection against MV stress in cells grown with and without nitrate (Raghavan et al. 2011). Interestingly, this single membrane-localized MnSOD protein has been shown to undergo post-translational modifications involving cleavage of membrane-targeting signal and linker peptides giving rise to active small-sized MnSOD isoforms of 27 and 24 kDa proteins. These small sized cytoplasmic MnSODs are produced irrespective of the nitrogen source provided for growth either in the form of homodimers or heterodimers in cells of this cyanobacterium. It has been proposed that the combined activities of the full-sized MnSOD and its derivatives may help combat $O_2^{\cdot-}$ produced in thylakoid lumen/periplasm and in cytosol, respectively, thereby enhancing the tolerance of *Anabaena* PCC 7120 to oxidative stress (Raghavan et al. 2015).

1.2.3 H_2O_2 production, toxicity and detoxification mechanisms:

Any increase in SOD activity inevitably leads to a parallel increase in H_2O_2 concentration within cell. In addition to SOD activity, incomplete oxidation of H_2O at the donor side of PSII also acts as a source of H_2O_2 in cyanobacteria, particularly under stress conditions (Pospíšil, 2009). Unlike $O_2^{\cdot-}$, H_2O_2 is more stable and has a relatively longer

half-life of approximately 1 millisecond. Once it accumulates at high concentration in cells, H_2O_2 impairs many metabolic activities, including photosynthesis. H_2O_2 distorts the structural integrity of phycobilisomes thus affecting light energy harvesting and light energy transfer to photosystems (Liu et al. 2005). It also affects the function of reaction center protein D1 of PSII leading to photoinhibition of photosynthesis. The translation elongation factor G (EF-G) is also a target of H_2O_2 in cyanobacteria (Nishiyama et al. 2011). Importantly, H_2O_2 along with $O_2^{\cdot-}$ and ferrous iron can trigger $HO^{\bullet}$ formation by Haber-Weiss and Fenton reaction. $HO^{\bullet}$ is the most destructive ROS, which cannot be scavenged by antioxidative enzymes. Thus, quick removal of ferrous iron and H_2O_2 is absolutely critical for survival of cyanobacteria.

The bacterioferritins and DNA-binding proteins from starved cells (DPS) belonging to ferritin family class of proteins sequester free cellular Fe^{2+} and store them in the form of Fe^{3+} in their ferroxidase centers by consuming O_2 or H_2O_2, leading to reduction in H_2O_2 levels and prevention of $HO^{\bullet}$ formation in cyanobacteria (Banerjee et al. 2013). H_2O_2 removal is catalyzed primarily by peroxidases, and a role for rubrerythrin in H_2O_2 detoxification has also been proposed in cyanobacteria. Among peroxidases, Peroxiredoxins (Prxs) are common, and occur in multiple numbers in cyanobacteria ranging from 3 in *Prochlorococcus marinus* SS120 to 12 in *Acaryochloris marina* MBIC 11017 (Bernroitner et al. 2009). The Prxs present in *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942, and *Anabaena* PCC 7120 have been biochemically characterized (Banerjee et al. 2013). Such Prxs include 1-Cysteine (Cys) Prx, typical 2-Cys Prx, atypical 2-Cys Prx with Type II Prx and Prx-Q as subgroups in these organsims. Prxs have been shown to have broad substrate specificity; in addition to reducing low

concentrations of H_2O_2 to water, Prxs also reduce peroxynitrite and alkyl hydroperoxides to nitrate and corresponding alcohols, respectively. The catalytic mechanism of all Prxs involves a catalytic cysteine (peroxidatic) which is oxidized following reaction with peroxide. The oxidized cysteine then forms a disulphide bond with another conserved cysteine (resolving). Depending upon the type of Prx, the disulphide bond may be intermolecular (typical 2-Cys Prx) or intramolecular (atypical 2-Cys Prx). The regeneration of reduced forms of Prxs, including 1-Cys Prx, which do not possess a resolving cysteine, is mediated by low molecular weight thiol proteins like glutathione, thioredoxin, glutaredoxin, or sulfiredoxin, which in turn derive electrons from photosynthetic electron transport activity (Bernroitner et al. 2009; Pérez-Pérez et al. 2009). The importance of Prxs in adaptation to oxidative and other environmental stress conditions have been revealed through transcripional, gene knockout and gene overexpression studies in various cyanobacteria (Perelman et al. 2003; Stork et al. 2005; Latifi et al. 2007). Further, localization studies have revealed that some of the Prxs are present in the cytoplasm, whereas others in the periplasm. In *Anabaena* PCC 7120, different Prxs have been found in heterocysts and vegetative cells, indication cell-specific role of Prx proteins in combating oxidative stress in filamentous cyanobacteria (Banerjee et al. 2013).

In addition to Prxs, genes highly homologous to glutathione peroxidase have been identified in some cyanobacteria (Tichy and Vermaas 1999; Bernroitner et al. 2009; Latifi et al. 2009; Pérez-Pérez et al. 2009). Two glutathione peroxidase-like-genes and their corresponding proteins have been biochemically characterized from *Synechocystis* sp. PCC 6803. These proteins have been shown to use NADPH as electron donor instead of

glutathione, and prevent membrane lipid peroxidation in *Synechocystis* sp. PCC 6803 during oxidative stress (Gaber et al. 2001, 2004). Apart from glutathione peroxidse like proteins, a novel heme-dependent peroxidase and a FNR-dependent peroxidase have been identified in *Anabaena* PCC 7120, and have been shown to play important roles in oxidative stress tolerance in this cyanobacterium. However, ascorbate-dependent peroxidase prevalent in plant chloroplasts has not been reported so far from cyanobacteria (Bernroitner et al. 2009; Latifi et al. 2009).

Catalase is another important class of hydroperoxidse, which efficiently decompose and detoxify high concentrations of H_2O_2 in most aerobic organisms. Unfortunately, catalase is not ubiquitous in cyanobacteria. Only some cyanobacteria (approximately 50 %) possess catalase (Bernroitner et al. 2009). The bifunctional heme-containing catalase-peroxidase (*katG*), and manganese (Mn)-containing monofunctional catalase are the major catalases identified in cyanobacteria. The monofunctional heme-containing catalase is rare in this class of microorganisms. KatG has been biochemically characterized from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942. The *katG* disrupted mutants of such species not only exhibit reduced ability to decompose high concentrations of H_2O_2, but also show decreased tolerance to exogenously applied H_2O_2 compared with their wild types (Tichy and Vermaas 1999; Perelman et al. 2003). The filamentous diazotrophs, for example, strains of *Nostoc* and *Anabaena*, however, do not possess *katG*, but possess genes for Mn-catalase (Bernroitner et al. 2009; Chakravarty et al. 2016; Hudek et al. 2017). Despite the coding potential for Mn-catalase, *Anabaena* sp. PCC 7120 (two isozymes) and *Anabaena variabilis* (one isozyme) either lack or display poor catalase activity, and is relatively more sensitive to H_2O_2 than *Synechocystis* sp. PCC

6803 (Bagchi et al. 1991; Pascual et al. 2010; Banerjee et al. 2012). On the other hand are cyanobacteria which are completely devoid of catalases, such as the unicellular non-diazotrophic marine strains of *Prochlorococcus* and the freshwater *Microcystis aeruginosa* (Bernroitner et al. 2009). The lack of catalase activity in *Prochlorococcus* has been cited as a major reason for its inability to grow as axenic cultures in laboratory and, its survival in natural habitat has been proposed to be dependent on the presence of H_2O_2-removing catalase-positive heterotrophic bacterial neighbors (Morris et al. 2011). *Microcystis aeruginosa* have also been shown to be highly sensitive to H_2O_2 (nearly ten times) than co-dwelling chlorophytes and diatoms in many natural fresh water ecosystems (Drábková et al. 2007 a, b; Mikula et al. 2014). Such a selective effect of H_2O_2 has led to its potential application as an effective and eco-friendly algaecide for controlling toxigenic *Microcystis* blooms in water bodies (Mikula et al. 2014; Piel et al. 2019). Overall, the previous studies indicate that many cyanobacteria may be particularly sensitive to the ROS H_2O_2, requiring further studies for selection or development of cyanobacterial strains with relatively higher ability to tolerate H_2O_2 for effective use in biotechnology.

1.3 Present study:

Keeping in view of the above, the present study was aimed at understanding the oxidative stress response of a photoautotrophic filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133. This cyanobacterium occurs in freshwater and terrestrial ecosystems. Although free-living, it can form symbiotic association with plants and fungi unlike many other cyanobacteria. It possesses multiple cell differentiation alternatives; motile hormogonia for infecting plants, akinetes (spore like structures) for surviving harsh environmental conditions, and heterocysts for N_2-fixation (Meeks et al. 2001). These

features make *Nostoc punctiforme* ATCC 29133 a high-value microorganism for agricultural, and biotechnological exploitation (Moirangthem et al. 2014; Moraes et al. 2017). This study details the ability of this cyanobacterium to deal with oxidative stress imposed by direct application of H_2O_2, *tert*-butyl hydroperoxide (an organic hydroperoxide mimic) and methyl viologen, a redox-cycling herbicide. Furthermore, *Anabaena* sp. PCC 7120, a phylogenetically close relative of *Nostoc punctiforme* ATCC 29133 was also included in this study to reveal differences, if any, between these two cyanobacteria in terms of their oxidative stress tolerance capacity.

CHAPTER 2

Materials and Methods

2.1 Organisms:

The organisms used in the present study are the filamentous, heterocystous, diazotrophic cyanobacteria *Nostoc punctiforme* strain ATCC 29133-S (hereafter *Nostoc* 29133) and *Anabaena* sp. PCC strain 7120 (hereafter *Anabaena* 7120).

2.2 Preparation of culture medium and sterilization:

The media used for growth of photoautotrophic *Nostoc* 29133 and *Anabaena* 7120 was BG11-medium (combined nitrogen source supplemented) containing 17.6 mM sodium nitrate buffered with equimolar 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) to pH 7.5 (Rippka et al. 1979). As and when required, the cultures were also grown in $BG11_0$-medium (wihout combined nitrogen source; N_2-medium). $BG11_0$-medium consisted of macronutrients (mM) and micronutrients (µM) dissolved in Millipore water. The macronutrients included Dipotassium hydrogen phosphate, 0.18; Sodium carbonate, 0.19; Magnesium sulphate, 0.30; Calcium chloride, 0.25; Ethylene diaminetetraacetic acid disodium salt, 0.003; Citric acid, 0.029; Ferric ammonium citrate, 0.030. The micronutrients included Boric acid, 46; Manganese chloride, 9.2; Zinc sulphate, 0.77; Sodium molybdate, 1.6; Copper sulphate, 0.32; Cobalt nitrate, 0.17. Prior to use for culturing, BG11- and $BG11_0$-media were sterilized by autoclaving at 121 °C (15 pounds per square inch) for 15 min.

2.3 Culture conditions:

The cultures of *Nostoc* 29133 and *Anabaena* 7120 were maintained on agar (1.5 % w/v) slants at room temperature (25 °C) and white light illumination (photon fluence rate 20-23 µmol m^{-2} s^{-1}) was provided continuously. The cultures were transferred from the slants aseptically in liquid BG11- or $BG11_0$-medium before experiments, and incubated as mentioned above.

2.4 Determination of growth:

Growth of *Nostoc* 29133 and *Anabaena* 7120 was determined by chlorophyll *a* (Chl *a*) content of cultures. For estimation of Chl *a*, 1 ml of cyanobacteria culture was centrifuged for 10 min at 2300×*g*, supernatant discarded. An equal volume of 100 % methanol was then added to the cell pellet and further incubated for 10 min at room temperature. Absorbance of the supernatant obtained after centrifugation (10 min at 2300×*g*) was measured at 663 nm using a Cary 60 spectrophotometer (Varian, USA), and the concentration of Chl *a* calculated as Chl *a* (µg ml^{-1}) = Absorbance at 663 nm×13.43 (Mackinney 1941).

2.5 Light microscopy:

The cyanobacterial cultures were examined under a light microscope (Olympus, Japan) at 400×magnification to determine the frequency of heterocysts, calculated as: Heterocyst frequency (%) = Number of heterocysts/Number of vegetative cells+Number of heterocysts×100

2.6 Measurement of Chl *a* fluorescence by pulse amplitude modulated (PAM) fluorometry:

The maximal efficiency of PSII photochemistry was determined as F_v/F_m in the cultures of *Nostoc* 29133 and *Anabaena* 7120 by using a Dual-PAM-100 fluorometer (Waltz, Effeltrich, Germany), where F_v was determined from F_m-F_0, where F_m and F_0 represent the maximal and minimal fluorescence yields in the dark adapted state, respectively (Schreiber et al. 1995). F_0 was determined in cultures after a dark adaptation period of 15 min followed by exposing the sample to a low-irradiance measuring light (0.24 µmol m^{-2} s^{-1}). Then, a saturating pulse of white light (1.6s, 1000 µmol m^{-2} s^{-1}) was applied to determine F_m.

2.7 Determination of total intracellular hydroperoxide:

To determine hydroperoxide content in cells, cultures of *Nostoc* 29133 and *Anabaena* 7120 were centrifuged at 2300×*g* for 10 min at room temperature. After discarding the supernatant, cultures were washed twice with sterile Millipore water. Then, cells equivalent to Chl *a* concentration of 1.5 µg ml^{-1} were mixed with 0.8 ml of methanol containing butylated hydroxytoluene (BHT, 0.01 %). To this reaction mixture, 0.1 ml of reagent A (2.5 mM ammonium ferrous sulphate, 0.25 mM sulphuric acid) was added followed by addition of 0.1 ml of reagent B (40 mM BHT, 1.25 mM xylenol orange in methanol), and incubated at room temperature for 30 min. The mixture was centrifuged at 2300×*g* for 10 min to pellet the cell debris. Absorbance of the supernatant was detrrmined at 560 nm, and the concentration of intracellular hydroperoxides was calculated using an extinction coefficient of 4.3×10^4 M^{-1} cm^{-1}, as described by Wolff (1994).

2.8 Extraction of cellular proteins:

To determine hydroperoxide content in cells, cultures of *Nostoc* 29133 and *Anabaena* 7120 were centrifuged at 2300×*g* for 10 min at room temperature. The cell pellet was washed twice with 36 mM potassium phosphate buffer (pH 7.4), following which the cell pellet was resuspended in the same buffer supplemented with protease inhibitor (phenylmethylsulphonyl fluoride, 1 mM) and cells broken in an ultra-sonicator (Soniprep 150 MSE) at 4 °C. The cell extracts were then centrifuged at 10000×*g* for 10 min at 4 °C, and total protein was estimated in the supernatant fraction.

2.9 Estimation of total protein:

Total protein concentration was measured by using a BioRad kit, according to manufacturer's instruction (Bradford 1976). 20 µl of supernatant fraction obtained as described above was mixed with 1 ml of Bradford reagent, and incubated in the dark for 5 min. Then, absorbance of reaction mixture was measured at 595 nm against a reagent blank. Protein concentration was determined from a calibration curve prepared using Bovine serum albumin (BSA) solution provided by the same manufacturer.

2.10 Preparation of non-denaturing gel for protein separation by electrophoresis:

The total proteins (25 µg) obtained as described above was separated by non-denaturing (native) polyacrylamide gel electrophoresis using a BioRad Mini gel apparatus (BioRad Laboratories, California, USA). The non-denaturing non-reducing gels (10 % resolving and 5 % stacking) contained 10 % glycerol, but no SDS. A 10 % resolving gel

was made by adding 2 ml of Millipore water, 1.6 ml of 30 % acrylamide and N,N'-methylenebisacrylamide mix (1:29), 1.3 ml of 1.5 M Tris buffer (pH 8.8), 0.05 ml of 10 % ammonium persulphate, and 0.002 ml of TEMED (Tetramethylethylenediamine). A 5 % stacking gel was made by adding 1.7 ml of Millipore water, 0.42 ml of 30 % acrylamide and N,N'-methylenebisacrylamide mix (1:29), 0.3 ml of 0.5 M Tris buffer (pH 6.8), 0.025 ml of 10 % ammonium persulphate, and 0.002 ml of TEMED. The sample loading dye contained 50 mM Tris buffer (pH 6.8), 0.1 % bromophenol blue and 10 % glycerol without β-mercaptoethanol, and electrophoresis buffer contained 250 mM Glycine and 25 mM Tris buffer (pH 8.3). Electrophoresis of protein samples was carried out at 4 °C for 3 h under constant amperage.

2.11 Determination of Superoxide dismutase (EC 1.15.1.1) activity in-gel:

After protein separation by electrophoresis, the gel was dipped in a staining solution made of 28 μM TEMED (N,N,N,N'- tetramethylethylenediamine) and 28 μM riboflavin in 36 mM potassium phosphate buffer (pH 7.8) for 30 min. The gel was stained for another 10 min in 2.5 mM nitrobluetetrazolium solution, and incubated under white light (20 μmol m^{-2} s^{-1}) for 15 min. The SOD activity was detected as colourless zone on purple-blue background of the gel (Beauchamp and Fridovich 1971). SOD isozymes were distinguished by soaking the gel for 30 min with 10 mM H_2O_2, prior to staining. The gel image was captured using a Gel documentation system (Syngene, Cambridge,UK).

2.12 Determination of Catalase (EC 1.11.1.6) activity in-gel:

Protein extraction, estimation and native PAGE was performed as described above. After electrophoresis, the gel was incubated with 10 mM H_2O_2 for 10 min, washed with Millipore water, and stained for catalase activity using 1 % each of ferric chloride and potassium ferricyanide solution. The catalase activity was detected as colourless zone on a greenish-yellow background of the gel (Weydert and Cullen 2010) and photographed.

2.13 Statistical analysis:

All experiments were replicated at least thrice. The results are reported as mean ± standard deviation. Student's *t*-test (Microsoft Excels version 10) was used to determine the significant differences between samples, and probability (P) values less than 0.05 were considered significantly different.

2.14 Chemicals:

The chemicals used in this study were high grade, and purchased from Himedia, SRL, and Merck, India.

CHAPTER 3

Differential catalase activity and tolerance to hydrogen peroxide in the filamentous cyanobacteria *Nostoc punctiforme* ATCC 29133 and *Anabaena* sp. PCC 7120

3.1 Introduction

Hydrogen peroxide (H_2O_2) is one of several reactive oxygen species (ROS) produced as a by-product of photosynthesis and/or respiratory processes in aerobic organisms (Latifi et al. 2009; Imlay 2013). The oxygen evolving photosynthetic cyanobacteria, major drivers of global carbon and nitrogen cycle and potential sources of biofuels and commodity chemicals, produce H_2O_2 mainly through superoxide dismutase (SOD) catalyzed disproportionation of superoxide radical ($O_2^{\bullet-}$), a by-product of photosynthetic electron transport activity (Banerjee et al. 2013; Singh et al. 2014; Kitchener and Grunden 2018). Incomplete oxidation of H_2O at the donor side of photosystem II (PSII) also generates H_2O_2 in cyanobacteria (Pospíšil 2009). A wide variety of naturally occurring stressors such as high light, ultraviolet rays, salinity, herbicides, heavy metals, high and low temperature etc. further increase the intracellular concentration of H_2O_2 in cyanobacteria (Latifi et al. 2009; Chauvat and Chauvat 2015; Mironov et al. 2019). Besides, cyanobacteria may also encounter H_2O_2 sourced from metabolic activities of other organisms or from photo-oxidation of chromophoric dissolved organic matter in their natural environments (Diaz and Plummer 2018; Zinser 2018).

While low-concentration H_2O_2 may function as a second messenger in cell signal transduction pathways, sufficiently high concentrations of H_2O_2 cause oxidative stress leading to loss of membrane integrity, destruction of light-harvesting pigments, impairment of PSII reaction center protein D1 and photosynthetic activity, and ultimately cell death in cyanobacteria (Drábková et al. 2007 a, b; Nishiyama et al. 2011; Mikula et al. 2012; Yang et al. 2018; Piel et al. 2019). It has been suggested that such adverse effects are most often not directly caused by H_2O_2, but rather by the ROS hydroxyl radical ($HO^{\bullet}$) formed from interaction of H_2O_2 with free intracellular ferrous iron via Fenton chemistry (Imlay 2013). Hence, cyanobacteria must promptly neutralize H_2O_2 to avoid formation of cell lethal $HO^{\bullet}$.

Cyanobacteria possess various antioxidative enzymes such as thiol-specific peroxiredoxins (Prxs) and catalases to neutralize H_2O_2 (Latifi et al. 2009; Banerjee et al. 2013). Whereas, Prxs reduce low concentrations of H_2O_2 (K_M in µM range), catalases efficiently decompose high concentrations of H_2O_2 (K_M in mM range) (Tichy and Vermaas 1999). Comparative genome sequence analysis in cyanobacteria has revealed that unlike Prxs, the distribution of catalases is not uniform; a large number of them (nearly 50 %) lack catalase-encoding gene (Bernroitner et al. 2009). Accordingly, several studies also revealed higher sensitivity to H_2O_2 in some cyanobacteria which lack catalase, such as the unicellular fresh water *Microcystis aeruginosa* and marine-dwelling *Prochlorococcus* (Morris et al. 2011; Mikula et al. 2012). Conversely, the ones containing catalase (heme-dependent KatG) like unicellular, non-diazotrophic cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 have been shown to tolerate high concentrations of H_2O_2 (Tichy and Vermaas 1999; Perelman et al. 2003). On the other hand, many KatG-

lacking filamentous diazotrophic cyanobacteria, particularly the species of *Anabaena*, which possess at least one catalase with manganese as cofactor (Mn-catalase), are reported to lack catalase activity, irrespective of whether they were grown in the presence or absence of H_2O_2 (Bagchi et al. 1991; Bernroitner et al. 2009; Banerjee et al. 2012b; Chakravarty et al. 2016; Ballal et al. 2020). Correspondingly, *Anabaena* sp. PCC 7120 despite possessing two Mn-catalases, was more sensitive to H_2O_2, as compared to *Synechocystis* sp. PCC 6803 (Pascual et al. 2010). A report also suggests that owing to higher basal level catalase activity *Synechococcus* sp. PCC 7942 is more tolerant to H_2O_2 than *Synechocystis* sp. PCC 6803 (Gupta and Ballal 2015). It is presently unclear whether variation in H_2O_2 tolerance also exists among filamentous cyanobacteria. Such information may be important, as this may lead to identification of stress resilient strains to be used in biotechnological applications (Kitchener and Grunden 2018).

In this study, H_2O_2 tolerance ability of *Nostoc punctiforme* ATCC 29133 (hereafter *Nostoc* 29133) was evaluated with respect to the reference strain *Anabaena* sp. PCC 7120 (hereafter *Anabaena* 7120) to determine possible differences, if any, between these two taxonomically closely related filamentous cyanobacteria. However, unlike *Anabaena* 7120, *Nostoc* 29133 has a symbiotic origin isolated from the cycad *Macrozamia* (Meeks et al. 2001; Campbell et al. 2008). *Nostoc* 29133 has previously been shown to adapt to a variety of stresses, including UVA radiation, herbicide methyl viologen and heavy metals (Soule et al. 2009; Moirangthem et al. 2014; Hudek et al. 2017). This cyanobacterium is also considered a potential candidate for production of biofuels and many other high-value compounds (Moraes et al. 2017). The findings presented in this study show that *Nostoc* 29133 is relatively more tolerant to H_2O_2 than *Anabaena* 7120 due to its higher intrinsic

constitutive H_2O_2 decomposition activity. This suggests that differences in H_2O_2 tolerance may exist even between closely related filamentous cyanobacteria. Further, the unique H_2O_2 stress-tolerant property of *Nostoc* 29133 is likely to add to its biotechnological value.

3.2 Materials and Methods:

3.2.1 Cyanobacterial strains and culture conditions: The batch cultures of *Nostoc* 29133 (*Nostoc punctiforme* strain ATCC 29133-S, also known as UCD 153, Campbell et al. 2008) and *Anabaena* 7120 (*Anabaena* sp. PCC strain 7120) were grown at 25 °C in BG11-liquid medium, pH 7.5, containing 17.6 mM sodium nitrate buffered with equimolar 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) from stock cultures maintained on slants containing BG11-solid medium with 1.5 % agar (Rippka et al. 1979). The cultures were continuously illuminated with cool fluorescent light (photon fluence rate of 20-23 µmol m^{-2} s^{-1}) during growth period, as described earlier (Moirangthem et al. 2014).

3.2.2 Determination of H_2O_2 tolerance: For H_2O_2 tolerance assay, the actively growing cultures, 100 ml each of *Nostoc* 29133 and *Anabaena* 7120, were pooled separately and washed twice with fresh BG11-medium. Such cultures were inoculated separately in 30 ml of fresh BG11-medium at equal chlorophyll *a* (Chl *a*) concentration. The H_2O_2 stock (30 % v/v; Merck, India) was diluted to 10 mM concentration with sterile Milli-Q water and subsequently different volumes of H_2O_2 were added to the cyanobacterial cultures at the start of the experiment to obtain concentrations ranging from 0-0.5 mM H_2O_2, and incubated as described above. Growth was monitored periodically by measuring the concentration of Chl *a* spectrophotometrically (Cary 60, Agilent, USA) in methanolic extracts of cyanobacterial cultures using absorbance value at 663 nm×13.43, where 13.43 represents extinction coefficient of Chl *a* (Mackinney 1941; Moirangthem et al. 2014).

3.2.3 Pulse amplitude modulated (PAM) fluorometry: The maximal efficiency of PSII photochemistry (F_v/F_m), which is a common and quick indicator of photosynthetic performance of cells, was measured in H_2O_2 treated and untreated cultures of both the cyanobacterial species after 15 min of dark adaptation using a Dual-PAM-100 fluorometer (Waltz, Effeltrich, Germany), as described earlier (Moirangthem et al. 2014). The F_v/F_m values were derived from $F_v = (F_m-F_0)$, where F_v represents the variable fluorescence signal, F_0 minimal fluorescence signal of dark-adapted cells, and F_m the maximal fluorescence signal after application of a saturating light pulse (Schreiber et al. 1995).

3.2.4 Determination of total intracellular peroxides: The total peroxide levels was measured in cyanobacterial cells after incubation in the presence of different concentrations of H_2O_2 (0-0.5 mM) for one day by a ferrous oxidation/xylenol orange (FOX) assay method as described earlier (Wolff 1994; DeLong et al. 2002; Moirangthem et al. 2014).

3.2.5 Determination of catalase activity: The cultures of *Nostoc* 29133 and *Anabaena* 7120 were exposed to increasing concentrations of H_2O_2 for a day. Such cells were centrifuged at 2300×*g* for 5 min, and the cell pellets obtained were washed twice with 36 mM potassium phosphate buffer (pH 7.4). The cell-free extracts were obtained by sonication of the cell pellets in the same buffer added with 1 mM protease inhibitor phenylmethylsulphonyl fluoride (PMSF), as described earlier (Moirangthem et al. 2014). The total protein in cell extracts was measured according to Bradford (1976). 50 µg of total protein was added to 50 mM phosphate buffer (pH 7) and 10 mM H_2O_2 in a final

volume of 1 ml. Catalase activity was determined by measuring the disappearance of H_2O_2 at 240 nm using an extinction coefficient of 43.6 M^{-1} cm^{-1} (Beers and Sizer 1952).

3.2.6 Statistical analysis: All experiments were performed at least three times and the results are presented as mean ± standard deviation. The significant differences (*P* values less than 0.05) were analyzed by Student's two-sample *t*-test (Microsoft Excels version 10) between control and H_2O_2 treated cyanobacterial cultures.

3.3 Results and Discussion

3.3.1 *Nostoc* 29133 exhibits higher tolerance to H_2O_2 than *Anabaena* 7120: Cyanobacteria are often exposed to H_2O_2 generated in surrounding environments (Diaz and Plummer 2018; Zinser 2018). Being a small and neutral molecule, extracellular H_2O_2 can readily cross cell membranes and enter into cells. At high concentrations, H_2O_2 can be highly damaging to cellular growth and metabolism, especially if there is free ferrous iron available in the cell since $HO^{\bullet}$ radicals will be formed by Fenton chemistry (Latifi et al. 2009; Banerjee et al. 2013; Imlay 2013). In order to reveal the potential differences in H_2O_2 tolerance of filamentous *Nostoc* 29133 and *Anabaena* 7120, the active cultures of both cyanobacterial strains were subjected to increasing concentrations of exogenously added H_2O_2 (0.1, 0.25, and 0.5 mM), and Chl *a* content of cultures was measured for 6 days. This range of H_2O_2 concentration was chosen based on studies in *Aphanizomonen ovalisporum*, a filamentous cyanobacterium, which fails to survive in the presence of 0.5 mM H_2O_2 (Kaplan-Levy et al. 2015). When incubated with 0.1 mM H_2O_2, Chl *a* content of *Nostoc* 29133 (Fig. 3.1A) and *Anabaena* 7120 (Fig. 3.1B) was inhibited more or less to similar extent compared to their respective controls. However, a distinct difference between the two strains was observed with increased doses of H_2O_2. Whereas, 0.25 and 0.5 mM H_2O_2 inhibited Chl *a* content of *Nostoc* 29133 by 12 to 20 % compared with untreated control (considered 100 %), similar treatments led to complete inhibition in *Anabaena* 7120. These results suggest that tolerance to H_2O_2 is higher in *Nostoc* 29133 compared to in *Anabaena* 7120, despite the two being taxonomically closely related to each other (order *Nostocales*) (Rippka et al. 1979). Differences in H_2O_2 tolerance among cyanobacteria have been described earlier, but such comparisons have been mostly

between different taxonomical groups, for example, comparisons of *Anabaena* 7120 and *Synechocystis* sp. PCC 6803 (Pascual et al. 2010), and of *Cylindrospermopsis* and *Planktothrix* (Yang et al. 2018). H_2O_2 tolerance seems to be a species dependent feature in cyanobacteria, and may be related to differential accumulation of H_2O_2 and/or $HO^{\bullet}$ (Drábková et al. 2007 a, b; Yang et al. 2018). This possibility was investigated by measuring the intracellular hydroperoxide concentrations in *Nostoc* 29133 and *Anabaena* 7120.

3.3.2 *Nostoc* 29133 displays lower intracellular peroxide levels than *Anabaena* 7120:

Total intracellular peroxide levels (includes lipid hydroperoxides) was determined in the cultures of *Nostoc* 29133 and *Anabaena* 7120 exposed to H_2O_2 for 1 day by FOX assay (Wolff 1994; DeLong et al. 2002). As shown in Fig. 3.2, incubation with 0.1 mM H_2O_2 barely affected the peroxide levels in either strain relative to their respective controls. However, exposure to higher concentrations of H_2O_2 led to differential increase in peroxide levels; *Nostoc* 29133 displayed considerably lower levels relative to *Anabaena* 7120. It is highly likely that relatively lower accumulation of peroxides within cells of *Nostoc* 29133 protected it from H_2O_2 lethality, as opposed to that in *Anabaena* 7120. To investigate if the ability to keep a low intracellular peroxide concentration is also shown in a lower damage to the metabolism, the inhibition of PSII activity was measured in *Nostoc* 29133 and *Anabaena* 7120.

3.3.3 *Nostoc* 29133 exhibits lower inhibition of PSII performance than *Anabaena* 7120:

PSII is a major target of H_2O_2 in many cyanobacteria (Drábková et al. 2007 a, b; Nishiyama et al. 2011; Mikula et al. 2012; Yang et al. 2018; Piel et al. 2019). The

maximum quantum yield of PSII (F_v/F_m), an indicator of PSII electron transport capacity, is a quick and sensitive parameter to assess PSII performance in cyanobacteria (Schreiber et al. 1995). Thus, F_v/F_m was measured in whole cells of *Nostoc* 29133 and *Anabaena* 7120 following 1 day treatment with increasing concentrations of H_2O_2 (Fig. 3.3). The H_2O_2 treated cultures (0.1 and 0.25 mM) of *Nostoc* 29133 did not show any appreciable change in F_v/F_m values compared to control cultures, though F_v/F_m was inhibited to a smaller extent in 0.5 mM treated cultures. In *Anabaena* 7120, treatment with 0.1 mM H_2O_2 resulted in a mild inhibition of F_v/F_m compared with control, however, higher concentrations completely inhibited F_v/F_m. These results suggest lower inhibition of PSII performance in *Nostoc* 29133 than in *Anabaena* 7120, which may be an effect of tighter regulation of intracellular peroxide levels in the former thus limiting free H_2O_2 to inhibit the PSII.

3.3.4 *Nostoc* 29133 possesses higher catalase activity than *Anabaena* 7120: To probe the underlying reason for differential accumulation of peroxides in *Nostoc* 29133 and *Anabaena* 7120, catalase activity was evaluated in the cell extracts of 1 day old H_2O_2-treated and untreated cultures. The antioxidative enzymes such as catalases and Prxs are known to participate in scavenging H_2O_2 in cyanobacteria (Tichy and Vermaas 1999; Perelman et al. 2003; Bernroitner et al. 2009; Latifi et al. 2009; Banerjee et al. 2013). However, unlike catalases, Prxs are susceptible to H_2O_2-mediated overoxidation and inactivation (Pascual et al. 2010). As shown in Fig. 3.4, catalase activity was nearly 20-fold higher in control cultures of *Nostoc* 29133 than that in control cultures of *Anabaena* 7120. While a 1.2-fold increase in catalase activity was observed in *Nostoc* 29133 after 0.25 mM H_2O_2 treatment, an approximately 3-fold increase in this activity was observed in

Anabaena 7120, compared to their respective controls. However, such an increase in catalase activity was clearly not enough to prevent death by H_2O_2 in *Anabaena* 7120 (Fig. 1B). As opposed to *Anabaena* 7120, a high intrinsic constitutive catalase activity seems to contribute to prompt and efficient decomposition of H_2O_2 resulting in lower intracellular hydroperoxide levels and higher tolerance to H_2O_2 in *Nostoc* 29133. This correlation of H_2O_2 tolerance and intrinsic catalase activity has also been demonstrated earlier in a comparative study between *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 (Gupta and Ballal 2015). It should be stressed that several cyanobacteria, including the filamentous forms, lack catalase activity and are sensitive to H_2O_2 (Bagchi et al. 1991; Bernroitner et al. 2009; Morris et al. 2011; Banerjee et al. 2012b; Mikula et al. 2012; Yang et al. 2018; Piel et al. 2019). In this context, *Nostoc* 29133 may be unique, as it possesses catalase activity and a superior ability to tolerate H_2O_2. The findings presented here should pave the way for further in-depth characterization of catalases present in this filamentous cyanobacterium and their roles in adaptation to H_2O_2 and other abiotic stress conditions, which presumably generate oxidative stress.

3.4 Conclusion: This study highlights differences in H_2O_2 tolerance between two closely related filamentous cyanobacteria *Nostoc* 29133 and *Anabaena* 7120. *Nostoc* 29133 exhibited lower inhibition of chlorophyll *a* and PSII performance, as compared to *Anabaena* 7120 in response to exogenous H_2O_2. The higher tolerance of *Nostoc* 29133 to H_2O_2 was accompanied by a tighter control of intracellular hydroperoxide level supported by higher intrinsic constitutive catalase activity, in contrast to that in *Anabaena* 7120. H_2O_2 stress tolerant photoautotroph like *Nostoc* 29133 is likely to be an important

biotechnological resource, and may be exploited as a potential source of valuable antioxidant catalase.

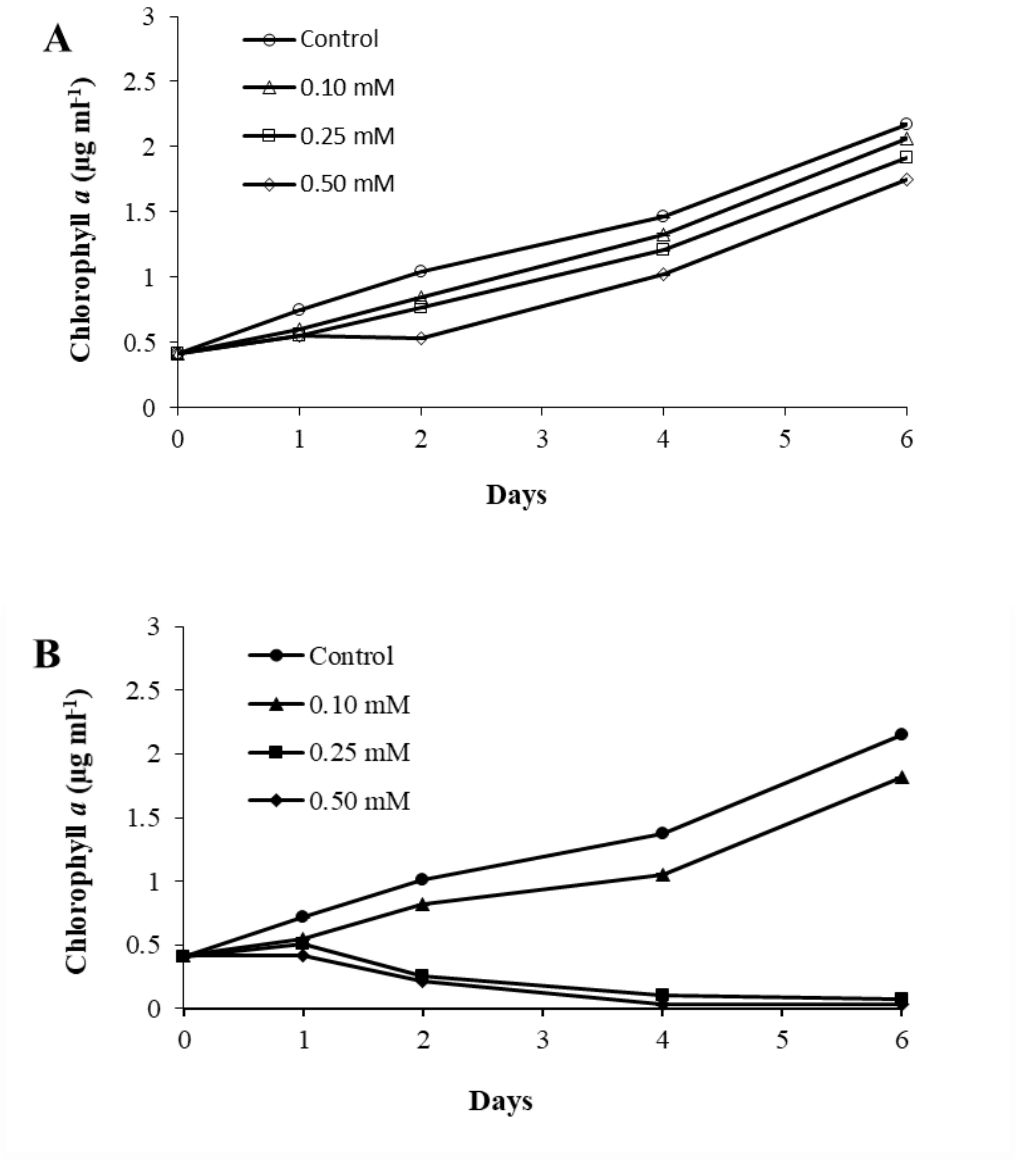

Fig. 3.1 Growth response of *Nostoc* 29133 and *Anabaena* 7120 to H_2O_2. The actively growing *Nostoc* 29133 (A) and *Anabaena* 7120 (B) cultures equivalent to 0.5 µg ml^{-1} of Chl *a* were seeded in fresh BG11-medium containing H_2O_2 (0-0.5 mM). The Chl *a* content of cultures was measured at periodic intervals for 6 days. The bars indicate standard deviation of three independent experiments, and are mostly smaller than the symbols

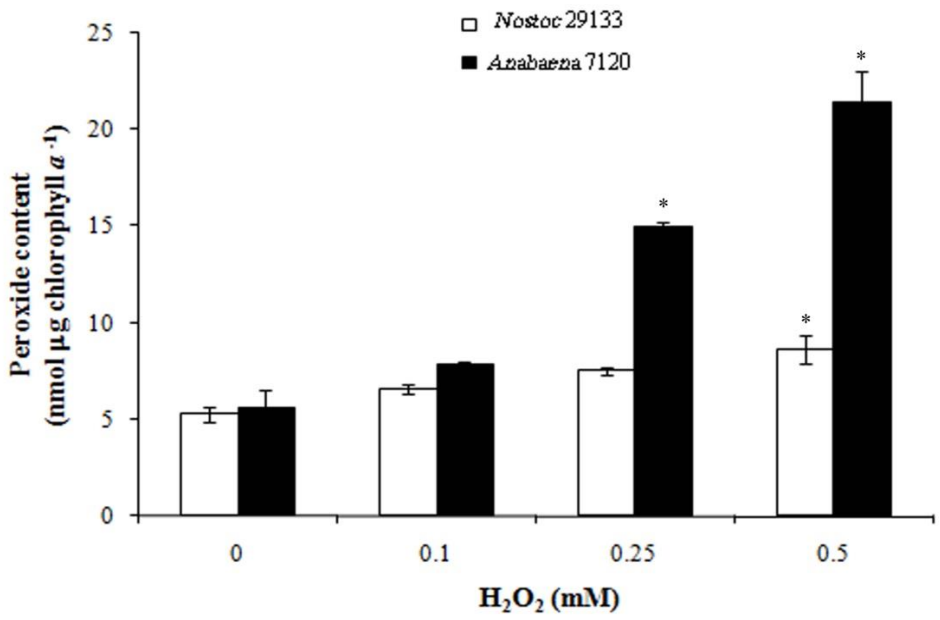

Fig. 3.2 Effect of H_2O_2 on total peroxide level in *Nostoc* 29133 and *Anabaena* 7120. The cyanobacterial cultures treated or untreated with H_2O_2 for 24 h in fresh BG11-medium were used to measure the total intracellular peroxide level by ferrous oxidation-xylenol orange method. The bars indicate standard deviation of three independent experiments. Asterisk (*, $P < 0.05$) on the bars represent significant difference as compared to respective control value

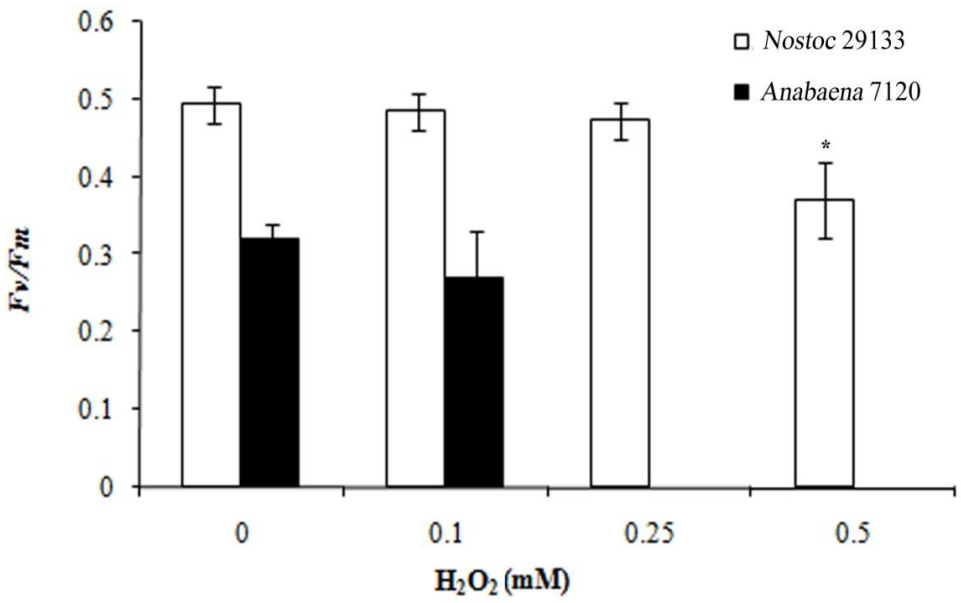

Fig. 3.3 Effect of H_2O_2 on maximum photochemical efficiency (*Fv*/*Fm*) of PSII in *Nostoc* 29133 and *Anabaena* 7120. The cyanobacterial cultures untreated or treated with different concentrations of H_2O_2 for 24 h in BG11-medium were harvested, washed and resuspended in the same medium. Such cultures (Chl *a* concentration 10 μg ml^{-1}) were dark adapted for 15 min followed by *Fv*/*Fm* measurement. The bars indicate standard deviation of three independent experiments. Asterisk (*, $P < 0.05$) on the bar represent significant difference as compared to respective control values

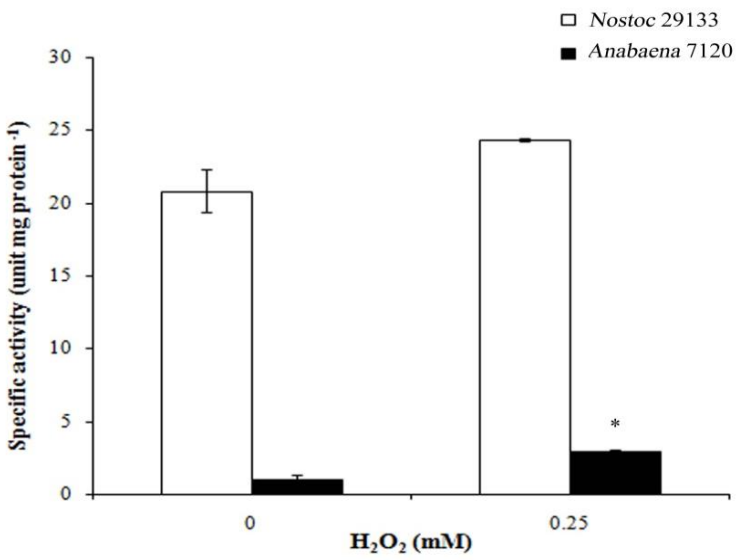

Fig. 3.4 Catalase activity assay in *Nostoc* 29133 and *Anabaena* 7120. The cyanobacterial cultures treated or untreated with H_2O_2 for 24 h in fresh BG11-medium were harvested, washed and resuspended in 36 mM phosphate buffer (pH 7.4) and broken by ultrasonication. The cell extracts equivalent to 50 µg total protein was used for measuring catalase activity. The bars indicate standard deviation of three independent experiments, and are sometimes smaller than the symbols. Asterisk (*, $P < 0.05$) on the bars represent significant difference as compared to respective control value

CHAPTER 4

Growth and antioxidative responses of the filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133 to *tert*-butyl hydroperoxide stress

4.1 Introduction

Organic hydroperoxides (ROOH) are reactive oxygen species (ROS) produced as by-products of aerobic metabolism in many organisms. Like other ROS, such as superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\bullet}$), ROOH can cause oxidative damage to vital biomolecules, including nucleic acids, proteins, and lipids when produced in excess (Halliwell and Gutteridge 1986; Latifi et al. 2009; Imlay 2013). The physiological and biochemical responses to organic hydroperoxide have been studied extensively in bacteria, yeast and mammalian cells using *tert*-butyl hydroperoxide (*t*-BOOH), a non-physiological analog of organic hydroperoxides (Aoshima et al. 1999; Sriprang et al. 2000; Alía et al. 2005; Patikarnmonthon et al. 2010). Externally applied *t*-BOOH generates *tert*-butyl alkoxyl and *tert*-butyl peroxyl radicals within cells via iron-catalyzed Fenton-type reaction. These radicals can further give rise to lipid alkyl radicals, which can cause peroxidation of lipids (polyunsaturated and monounsaturated fatty acids) in cell membranes (Halliwell and Gutteridge 1986). Unless detoxified quickly, *t*-BOOH can perturb the structure and function of cell membranes, and kill cells.

The oxygen-evolving photosynthetic cyanobacteria are considered as excellent model systems for studying oxidative stress tolerance mechanisms (Banerjee et al. 2013).

Protective strategies against oxidative stress have been studied in details in various cyanobacterial species by exposing them to methyl viologen, a redox-cycling herbicide (accelerates $O_2^{\bullet-}$ production within cells by abstracting electrons from photosystem I followed by oxygen reduction) or H_2O_2 (Latifi et al. 2009). Such studies have highlighted the importance of different antioxidative enzymes, including superoxide dismutases (SODs; catalyze disproportionation of $O_2^{\bullet-}$), and H_2O_2 eliminating catalases and/or peroxiredoxins (Prxs) in cellular protection against $O_2^{\bullet-}$ and H_2O_2 assault (Latifi et al. 2009; Banerjee et al. 2013) However, there are limited studies on the response of cyanobacteria to ROOH-induced oxidative stress, restricted only to the unicellular freshwater *Synechocystis* sp. PCC 6803 and the filamentous diazotrophic *Anabaena* sp. PCC 7120 (Maeda et al. 2005; Banerjee et al. 2013; Kammerscheit et al. 2019).

Similar to heterotrophic organisms, cyanobacteria also seems to be sensitive to *t*-BOOH. The sensitivity of *Synechocystis* sp. PCC 6803 to *t*-BOOH has been ascribed to increased production of malondialdehyde, a product of lipid peroxidation, extremely damaging to membrane integrity (Maeda et al. 2005; Kammerscheit et al. 2019). A membrane-associated glutathione-S-transferase (GST), certain Prxs (reduce *t*-BOOH to alcohol), and glutathione peroxidase-like proteins have been implicated in detoxification of *t*-BOOH in this cyanobacterium (Gaber et al. 2001, 2004; Pérez-Pérez et al. 2009; Kammerscheit et al. 2019). Although no sensitivity test has been reported, increased expression of some Prxs has also been noted in *Anabaena* sp. PCC 7120 upon exposure to *t*-BOOH (Cha et al. 2007; Banerjee et al. 2012a, 2013). The responses of other important antioxidative enzymes (for example, SODs and catalases) to oxidative stress induced by *t*-BOOH have not been described for cyanobacteria.

Nostoc punctiforme ATCC 29133 (hereafter *Nostoc* 29133) is an ecologically and economically important filamentous cyanobacterium (Meeks et al. 2001). This photoautotroph possesses multiple antioxidative enzymes, including SODs, Prxs and catalases (Bernroitner at al. 2009). Previous studies revealed that this cyanobacterium is able to adapt to oxidative stress induced by MV and H_2O_2 (Moirangthem et al. 2014). In this study, the response of *Nostoc* 29133 to *t*-BOOH was characterized to gain a better insight into its oxidative stress tolerance mechanisms. The results show that *t*-BOOH induced oxidative stress and inhibited growth in *Nostoc* 29133 by enhancing hydroperoxide production in a concentration-dependent manner, and the ability to tolerate *t*-BOOH was dependent on *t*-BOOH detoxification capacity of cells. Although *t*-BOOH exposure did not affect SOD activity, a new catalase isoenzyme was induced. The results further demonstrate that *Anabaena* sp. PCC 7120 exhibit similar sensitivity to *t*-BOOH as *Nostoc* 29133, implying that closely related filamentous cyanobacteria may possess similar mechanisms to tackle *t*-BOOH stress.

4.2 Materials and Methods

4.2.1 Cyanobacterial strains and culture conditions: The batch cultures of *Nostoc* 29133 (*Nostoc punctiforme* strain ATCC 29133-S) were grown at 25 °C in BG11-liquid medium, pH 7.5, containing 17.6 mM sodium nitrate buffered with equimolar 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) from stock cultures maintained on slants containing BG11-solid medium with 1.5 % agar (Rippka et al. 1979). When required *Anabaena* sp. PCC 7120 (hereafter *Anabaena* 7120) was also grown in the same medium as *Nostoc* 29133. The cultures were continuously illuminated with cool fluorescent light (photon fluence rate of 20-23 µmol m^{-2} s^{-1}) during growth period, as described earlier (Moirangthem et al. 2014).

4.2.2 Determination of *tert*-butyl hydroperoxide (*t*-BOOH) tolerance: For *t*-BOOH tolerance assay, the actively growing cultures of *Nostoc* 29133 and *Anabaena* 7120 (100 ml each) were pooled and washed twice with fresh BG11-medium. Such cultures were inoculated separately in 30 ml of fresh BG11-medium at equal chlorophyll *a* (Chl *a*) concentration. The *t*-BOOH solution (70 % v/v; Merck, India) was diluted with sterile Milli-Q water and subsequently different volumes of *t*-BOOH were added to the cyanobacterial cultures at the start of the experiment to obtain concentrations ranging from 0-100 µM, and incubated as described above. Growth was monitored periodically by measuring the concentration of Chl *a* in cyanobacterial cultures. Chl *a* was determined in the methanolic extracts by measuring absorbance at 663 nm×13.43, where 13.43 represents extinction coefficient of Chl *a* (Mackinney 1941).

4.2.3 Determination of total intracellular peroxides: The total peroxide levels was measured in the cultures of *Nostoc* 29133 after incubation in the presence of different concentrations of *t*-BOOH (0-100 µM) for one day by a ferrous oxidation-xylenol orange (FOX II) assay method as described earlier (Wolff 1994; DeLong et al. 2002; Moirangthem et al. 2014).

4.2.4 In-gel determination of SOD and catalase activity: The cultures of *Nostoc* 29133 were exposed to increasing concentrations of *t*-BOOH (0-100 µM) for a day. Such cells were centrifuged at 2300×*g* for 5 min, and the cell pellets obtained were washed twice with 36 mM potassium phosphate buffer (pH 7.4). The cell-free extracts were obtained by sonication of the cell pellets in the same buffer added with 1 mM protease inhibitor phenylmethylsulphonyl fluoride (PMSF), as described earlier (Moirangthem et al. 2014). The total protein in cell extracts was measured according to Bradford (1976). Equal amounts of total protein from samples were separated on 12 % non-denaturing polyacrylamide gels (Laemmli 1970), following which the gels were stained separately for SOD activity according to Beauchamp and Fridovich (1971) and catalase activity according to Weydert and Cullen (2010). The activity bands were visualized as achromatic areas on gels.

4.2.5 Determination of *t*-BOOH detoxification activity: *t*-BOOH detoxification activity was assessed by FOX I method with minor modifications (Wolff 1994). Briefly, the exponentially growing cultures of *Nostoc* 29133 were harvested, washed twice with 50 mM HEPES buffer (pH 7), and the whole cells were suspended in the same buffer at a Chl

a concentration of 3.5 µg Chl *a* ml^{-1} and incubated at 25 °C illuminated with 30 µmol m^{-2} s^{-1} light during the whole experimental period. The assay was initiated by adding 50 or 100 µM *t*-BOOH at time 0 to such cultures. Aliquots of samples were withdrawn at regular time intervals, mixed and incubated for 30 min in the presence of FOX I reagent followed by centrifugation at 10000×*g* for 5 min. The absorbance of the supernatant fractions was measured at 560 nm and the residual amount of *t*-BOOH was calculated from a standard curve prepared with known concentrations of *t*-BOOH.

4.2.6 Statistical analysis: All experiments were performed at least thrice and the results are presented as mean ± standard deviation. The significant differences (*P* values less than 0.05) were analyzed by Student's two-sample *t*-test (Microsoft Excels version 10) between control and *t*-BOOH treated cyanobacterial cultures.

4.3 Results and Discussion

4.3.1 Growth response of *Nostoc* 29133 to *t*-BOOH: Being an amphiphilic oxidant, *t*-BOOH can readily cross cell membranes and enter cells. To evaluate growth response to this oxidant, cultures of *Nostoc* 29133 were exposed to increasing concentrations of exogenously added *t*-BOOH (50, 75 and 100 µM) in BG11-medium, and the growth was monitored by measuring Chl *a*. As shown in Fig. 4.1A, growth of *Nostoc* 29133 was inhibited by 8 % when incubated with 50 µM *t*-BOOH compared to *t*-BOOH-untreated control (100 %) after 6 days. A 58 % growth inhibition was noted when *Nostoc* 29133 was incubated with 75 µM *t*-BOOH, and 77 % with 100 µM *t*-BOOH during the same time period. These results suggest that *Nostoc* 29133 is sensitive to *t*-BOOH, and this sensitivity is dose-dependent in nature, as has also been reported previously in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Maeda et al. 2005; Kammerscheit et al. 2019). Earlier studies have shown that cyanobacterial species often differ with regard to their sensitivity to various stressors (Pascual et al. 2010; Ballal and Gupta 2015). For instance, *Nostoc* 29133 and *Anabaena* sp. PCC 7120 vary in terms of their sensitivity to H_2O_2 (Chapter 3). To determine whether they are also differently sensitive to *t*-BOOH, *Anabaena* 7120 was tested for its growth response to *t*-BOOH (Fig. 4.1B). The result shows that *t*-BOOH sensitivity of *Anabaena* 7120 is similar to that observed in *Nostoc* 29133, implying that these two closely related filamentous cyanobacteria may have similar mechanisms to deal with *t*-BOOH induced stress. To analyze the basis of sensitivity, *t*-BOOH detoxification capacity of *Nostoc* 29133 was determined.

4.3.2 *t*-BOOH detoxification activity of *Nostoc* 29133: Exponentially growing cultures of *Nostoc* 29133 (*t*-BOOH untreated) were exposed to 50 µM, a concentration which causes mild growth inhibition, and 100 µM *t*-BOOH separately, and *t*-BOOH detoxification activity was studied over time (Fig. 4.2). While *Nostoc* 29133 detoxified 72 % of 50 µM *t*-BOOH within 30 min of experimental period, only 58 % of 100 µM *t*-BOOH could be detoxified by this cyanobacterium during the same time period. In cyanobacteria, including *Nostoc* 29133, Prxs detoxify *t*-BOOH by reducing it to less-toxic alcohol (Banerjee et al. 2012a, 2013). The slower detoxification of relatively high concentration of *t*-BOOH (100 µM) observed in *Nostoc* 29133 could possibly be due to lack of Prxs and/or inhibition of activity. The inhibition may result from shortage of reducing equivalents supplied by photosynthetic electron transport chain and/or the availability of small thiol-containing molecules, such as thioredoxin, glutaredoxin, and glutathione etc, which ferries electrons to Prxs (Bernroitner et al. 2009). Indeed, this limitation of Prxs in reducing high concentrations of H_2O_2 has been demonstrated earlier (Pascual et al. 2010). Although mechanisms concerning *t*-BOOH detoxification remain to be examined in future, an increase in residence time of *t*-BOOH within cells due to delayed detoxification may lead to enhanced formation of intracellular hydroperoxides, including lipid hydroperoxides in *Nostoc* 29133. This hypothesis was tested by determining hydroperoxide content in cells of *Nostoc* 29133.

4.3.3 Total hydroperoxide content in *Nostoc* 29133: The FOX assay is a commonly used method for measurement of peroxides (H_2O_2 and ROOH) in whole cells (Do TQ et al. 1996; Sakamoto et al. 1998). The total intracellular hydroperoxide content was measured in the cultures of *Nostoc* 29133 treated or untreated with different

concentrations of *t*-BOOH for one day (Fig. 4.3). The control culture exhibited a hydroperoxide content of 6.6 nmol ug Chl a^{-1}, considered 100 %. After treatment with 75 and 100 µM of *t*-BOOH, the hydroperoxide levels rose by 117 and 143 %, respectively, compared to control cultures. These results suggest that *t*-BOOH exposure leads to increased production of hydroperoxides, including lipid hydroperoxides in cells of *Nostoc* 29133. These observations are consistent with slower detoxification of 100 µM *t*-BOOH and consequent sensitivity to this oxidant. Previous studies on yeast, bacteria, and mammalian cells have shown that in addition to Prxs, other important antioxidative enzymes, such as SODs and catalases also participate in managing *t*-BOOH induced oxidative stress (Aoshima et al. 1999; Alía et al. 2005; Gazdag et al. 2014). Thus, SOD activity was determined in cells of *Nostoc* 29133 to analyze its possible role during *t*-BOOH stress.

4.3.4 SOD activity in *Nostoc* 29133: SOD executes disproportionation of $O_2^{\bullet-}$ to H_2O_2 and O_2 (Latifi et al. 2009; Imlay 2013). *Nostoc* 29133 possesses three putative SOD isoenzymes; two membrane-localized MnSODs (SodA) and a cytosolic FeSOD (SodB) (Moirangthem et al., 2014). SOD activity was assessed by non-denaturing gel electrophoresis (native PAGE) in cell extracts of *Nostoc* 29133 grown for a day in the absence (control) and presence of 75 and 100 µM *t*-BOOH (Fig. 4.4A and B). The control culture of this cyanobacterium displayed two major and one minor SOD activity bands in-gel. The major SOD activity band was identified as FeSOD (fast-migrating and relatively more sensitive to H_2O_2 judged by decrease in band intensity as compared to its H_2O_2 untreated control, compare Fig. 4.4A and B) and MnSOD (slow-migrating and relatively more tolerant to H_2O_2 judged by similar band intensity as compared to its H_2O_2 untreated

control). The minor SOD activity band could be a chimera of Fe and MnSOD, as it was only partially sensitive to H_2O_2 (Moirangthem et al. 2014). In cultures treated with *t*-BOOH, the band intensities corresponding to FeSOD and MnSOD activities were similar to control cultures, suggesting that $O_2^{\bullet-}$ dismutation ability of this cyanobacterium is not affected by exposure to *t*-BOOH. However, it is interesting to note that *t*-BOOH does not inhibit FeSOD activity, unlike methyl viologen ($O_2^{\bullet-}$ producer) and H_2O_2, indicating that the cellular response to different oxidants may vary in *Nostoc* 29133 (Moirangthem et al. 2014). Given that SOD activity leads to formation of H_2O_2, which is detoxified by catalase and/or Prxs, the activity of the former was evaluated in *Nostoc* 29133.

4.3.5 Catalase activity in *Nostoc* 29133: Catalase executes decomposition of H_2O_2 to H_2O and O_2 (Banerjee et al. 2013). *Nostoc* 29133 possesses three putative catalases; one heme-dependent and the other two dependent on manganese (Mn-containing catalase) (Bernroitner et al. 2009). Catalase activity was assessed by non-denaturing gel electrophoresis (native PAGE) in *Nostoc* 29133 grown for a day in the absence (control) and presence of *t*-BOOH 75 and 100 µM (Fig. 4.5). The control culture of this cyanobacterium displayed two catalase activity bands in-gel. The intensity of these two catalase activity bands was similar in *t*-BOOH treated cells. A third catalase activity band was induced in *t*-BOOH treated cells, the intensity of which did not change with increase in *t*-BOOH concentration. Although the identity of these catalases could not be determined in this study, the results suggest involvement of catalase activity in combating *t*-BOOH in *Nostoc* 29133, which is intriguing, as catalases are not known to detoxify *t*-BOOH. As mentioned above, detoxification of *t*-BOOH in cyanobacteria is carried out mainly by Prxs, which also detoxifies H_2O_2, the latter being also the substrate for catalase. It is

possible that while *t*-BOOH is being detoxified by Prxs, SOD-derived H_2O_2 escapes being detoxified by Prxs only to be decomposed by catalase. This could be a plausible reason for increased catalase activity observed in *Nostoc* 29133 upon *t*-BOOH treatment.

4.4 Conclusion: The present study demonstrates that filamentous cyanobacterium *Nostoc* 29133 and closely related *Anabaena* 7120 are equally sensitive to organic hydroperoxide analog *t*-BOOH. The increased accumulation of intracellular hydroperoxides in *Nostoc* 29133 resulted from inability of this cyanobacterium to quickly and completely detoxify *t*-BOOH, presumably via activity of peroxiredoxins, and this could well be the basis for its sensitivity to *t*-BOOH. The activity of antioxidative enzyme superoxide dismutase was not influenced by *t*-BOOH, but a new catalase isoenzyme was induced in response to this oxidant in *Nostoc* 29133. These findings suggest that in addition to *t*-BOOH detoxifying peroxiredoxins, catalase activity may also be important to tackle *t*-BOOH induced oxidative stress in *Nostoc* 29133. Further studies using knockout mutants may be necessary to determine the precise roles of each of these enzymatic antioxidants in mitigating organic hydroperoxide stress in cyanobacteria.

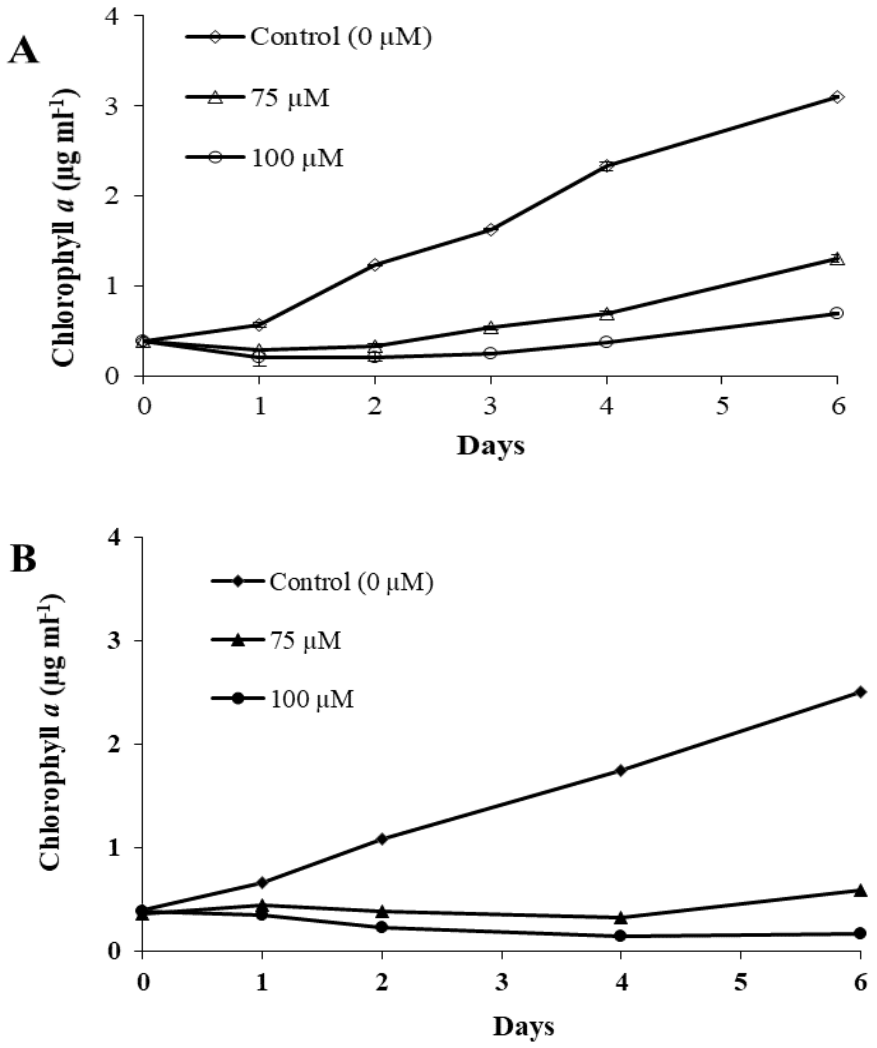

Fig. 4.1 Growth of *tert*-Butyl hydroperoxide-exposed and control cultures of *Nostoc* 29133 (A) and *Anabaena* 7120 (B). Exponentially growing cultures of cyanobacteria were harvested and washed twice with fresh BG11-medium, and such cultures were transferred at equal density to fresh medium added with increasing concentrations of *tert*-butyl hydroperoxide. Growth was measured at regular intervals by determining Chl *a* content. Depicted error bars mostly smaller than the symbols, represent standard deviation of three independent experiments

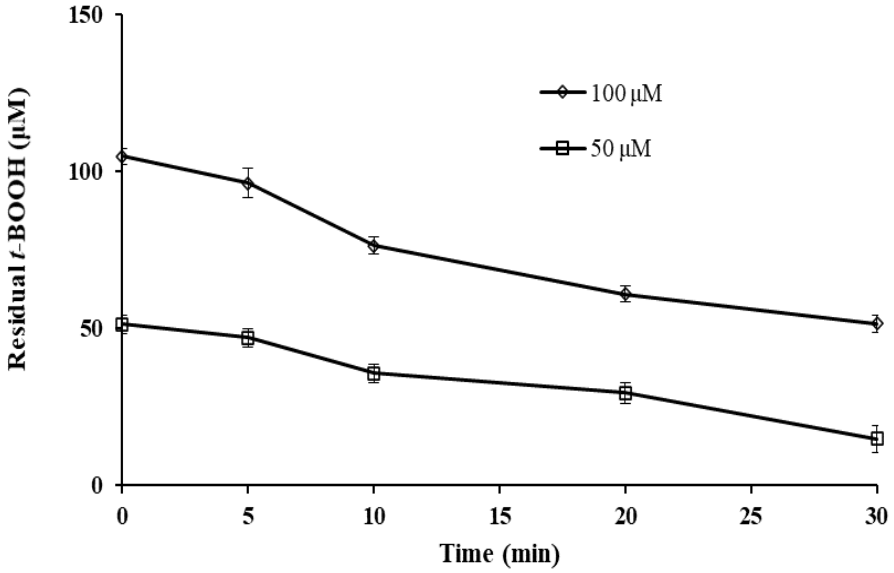

Fig. 4.2 *tert*-Butyl hydroperoxide detoxification activity in *Nostoc* 29133. The actively growing cultures in BG11-medium were harvested, washed and suspended in 50 mM HEPES buffer at a Chl *a* concentration of 3.5 µg ml^{-1}. *t*-BOOH (50 and 100 µM final concentration) was then added to separate cultures at time 0 and incubated under light. Aliquots of cell suspensions were withdrawn at periodic intervals and remaining *tert*-butyl hydroperoxide measured by ferrous oxidation-xylenol orange method. The bars indicate standard deviation of three independent experiments

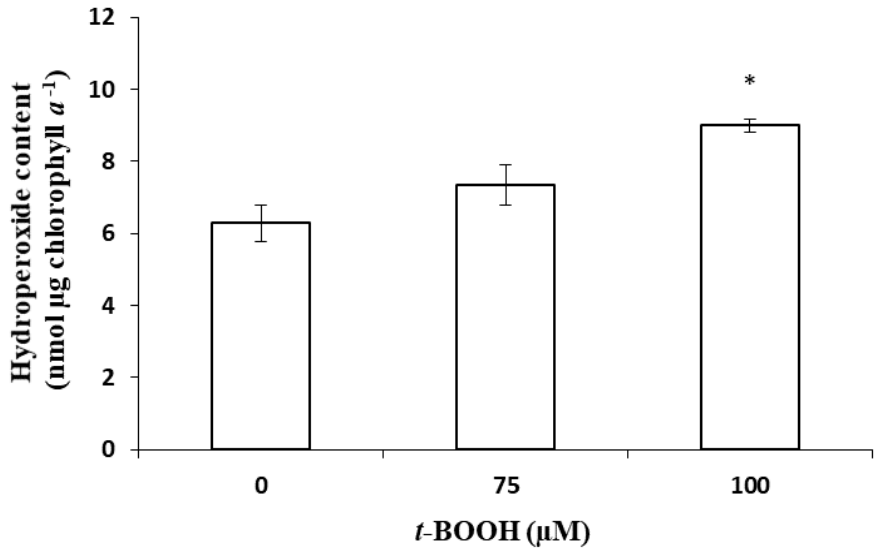

Fig. 4.3 Total hydroperoxide levels in *tert*-Butyl hydroperoxide-exposed and control *Nostoc* 29133. Exponentially growing cultures of *Nostoc* 29133 were incubated for 1 day at equal Chl *a* density (0.5 µg ml^{-1}) with indicated concentrations of *tert*-butyl hydroperoxide in fresh BG11-medium, and such cultures were washed twice with 36 mM phosphate buffer (pH 7.8) followed by measurement of total hydroperoxide levels in cell pellets by ferrous iron oxidation-xylenol orange (FOX) assay. Depicted error bars represent standard deviation of three independent experiments

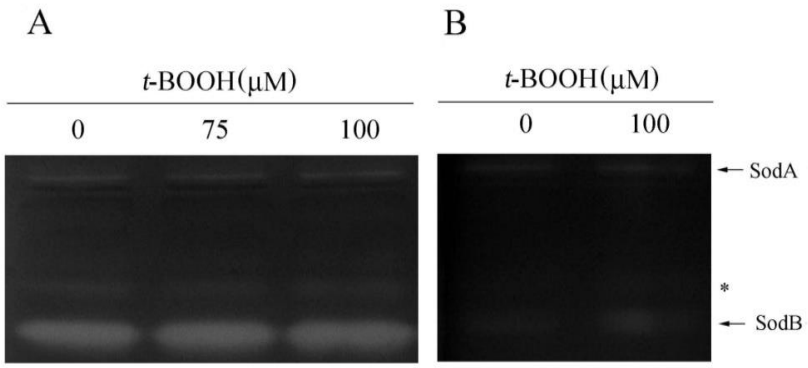

Fig. 4.4 In-gel superoxide dismutase activity of *tert*-Butyl hydroperoxide-exposed and control *Nostoc* 29133. The cyanobacterial cultures treated or untreated with *ter*-butyl hydroperoxide for 1 day in fresh BG11-medium were harvested, washed and resuspended in 36 mM phosphate buffer (pH 7.4) and broken by ultrasonication. The cell extracts equivalent to 20 µg total protein were separated by native PAGE (12 % gel) and stained for catalase activity. Asterisk represent SOD chimera. Similar results were obtained from two independent experiments, and a representative gel is shown

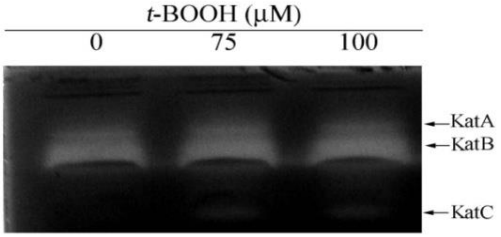

Fig. 4.5 In-gel catalase activity in *tert*-Butyl hydroperoxide-exposed and control *Nostoc* 29133. The cyanobacterial cultures treated or untreated with ter-butyl hydroperoxide for 1 day in fresh BG11-medium were harvested, washed and resuspended in 36 mM phosphate buffer (pH 7.4) and broken by ultrasonication. The cell extracts equivalent to 25 µg total protein were separated by native PAGE (12 % gel) and stained for catalase activity. Similar results were obtained from two independent experiments, and a representative gel is shown

CHAPTER 5

Growth on nitrate improves tolerance of filamentous cyanobacterium *Anabaena* sp. PCC 7120 to the redox-cycling herbicide methyl viologen

5.1 Introduction

Photoautotrophic filamentous cyanobacteria, such as the species of *Nostoc* and *Anabaena* are important components of microbial population in many diverse terrestrial and aquatic ecosystems, including in tropical wetland rice fields, where they contribute significantly to soil fertility (Singh et al. 2016). These cyanobacteria use atmospheric dinitrogen (N_2-fixation) or various fixed-nitrogen sources such as ammonium, nitrate etc. as nitrogen-nutrient to sustain photoautotrophic growth (Muro-Pastor et al. 2005; Frias and Flores 2015). The chemical nitrogen fertilizers often added to rice fields to augment plant growth is a major source of fixed-nitrogen for co-dwelling cyanobacteria. During growth on fixed nitrogen sources, heterocyst (site for nitrogenase localization) formation and N_2-fixation is inhibited in filamentous cyanobacteria (Bhattacharya et al. 2002; Frias and Flores 2015). Herbicides used frequently in rice fields also hamper N_2-fixation and inhibit cyanobacterial growth (Gomes and Juneau 2017). Methyl viologen (MV; 1,1'-dimethyl-4, 4'-bipyridinium dichloride), also called as paraquat, is one such herbicide (Raghavan et al. 2011; Moirangthem et al. 2014; Panda et al. 2015).

MV readily entry into cells of cyanobacteria, following which it undergoes reduction by accepting an electron from the acceptor side of photosystem (PS) I, including from PSI-reduced ferredoxin, and thereby gets converted into monovalent cation (Fujii et

al. 1990; Blot et al. 2011; Sétif 2015). Subsequently, this monovalent cation is reoxidized by donating electron to oxygen giving rise to toxic reactive oxygen species (ROS) superoxide radical anion ($O_2^{\bullet-}$). Repeated cycles of MV reduction and oxidation increases the intracellular level of $O_2^{\bullet-}$, which further gives rise to other ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) (Babbs et al. 1989). Protection from MV-induced ROS is facilitated by several ROS-scavenging antioxidative enzymes, such as superoxide dismutase (SOD), catalases and peroxiredoxins (Prxs) in cyanobacteria (Thomas et al. 1998; Cha et al. 2007; Latifi et al. 2007; Zhao et al. 2007; Raghavan et al. 2011, 2015; Banerjee et al. 2012 a, b, 2013; Moirangthem et al. 2014; Sae-Tang et al. 2016). However, when cellular antioxidative systems are overwhelmed due to excessive production of ROS, vital biomolecules such as nucleic acids, proteins, and lipids are destroyed, eventually leading to cell death (Latifi et al. 2009).

Reported evidence indicates that light intensity, a commonly occurring environmental variable, influences MV toxicity in cyanobacteria (Blot et al. 2011). High light intensity aggravates MV toxicity in the marine cyanobacterium *Synechococcus* sp. WH 7803. This effect of high light on MV toxicity has been attributed to increased electron transport activity at PSI complex leading to fast and continuous leakage of electrons to MV causing increased production of cell-toxic ROS. Although cells grown in low light intensity are also sensitive to MV, relatively lower photosynthetic electron transport activity in such cells leads to decreased formation of ROS, and lower toxicity, as compared to high light grown cells (Blot et al. 2011). Thus, one would expect that diverting photosynthetically generated electrons away from MV towards other metabolic pathways may constitute a possible alternate mechanism preventing MV induced ROS

production and toxicity. It is well established that many metabolic pathways including nitrate assimilation is fed by electrons arising from photosynthetic electron transport activity (Chauvat and Chauvat 2014; Frias and Flores 2015). Nitrate assimilation in filamentous cyanobacteria is nitrate-inducible. The conversion of nitrate to nitrite is catalyzed by nitrate reductase consuming two electrons during this process, and subsequently nitrite is converted to ammonium by nitrite reductase consuming six electrons. In this way nitrate assimilation pathway consumes eight electrons per nitrogen atom assimilated (Frias and Flores 2015). Thus this pathway is considered an important electron sink in cyanobacteria with proposed role in balancing redox-state of cells (Klotz et al. 2015). Based on this knowledge, it was hypothesized that if nitrate assimilation pathway can compete with MV for photosynthetically generated electrons, this may prevent or slow down electron flow to MV, and thereby decrease ROS formation and resultant toxicity to cyanobacteria.

To evaluate this possibility, MV sensitivity of filamentous N_2-fixing cyanobacterium *Anabaena* sp. PCC 7120 (hereafter *Anabaena*), was compared in terms of chlorophyll *a* content, ROS formation, antioxidative catalase activity under nitrate-sufficient and deficient growth conditions during the present study. The results revealed that growth on nitrate provides better tolerance to MV in *Anabaena* than when it is grown in the absence of nitrate. A similar protective effect of nitrate addition on MV tolerance was also detected in the phylogenetically related filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133. These findings implicate a conserved role for nitrate in reducing MV sensitivity in filamentous cyanobacteria.

5.2 Materials and Methods

5.2.1 Cyanobacterial strains and culture conditions: The batch cultures of *Anabaena* 7120 (*Anabaena* sp. PCC strain 7120) were grown at 25 °C in BG11 medium, pH 7.5, with 17.6 mM sodium nitrate buffered with equimolar 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) or without nitrate ($BG11_0$; N_2-medium) (Rippka et al. 1979). When required *Nostoc* 29133 (*Nostoc punctiforme* strain ATCC 29133-S) was also grown in the same medium as *Anabaena* 7120. The cultures were continuously illuminated with cool fluorescent light (photon fluence rate of 20-23 µmol m^{-2} s^{-1}) during growth period, as described earlier (Moirangthem et al. 2014).

5.2.2 Determination of Methyl viologen (MV) tolerance: For MV tolerance assay, the actively growing cultures, 100 ml each of *Anabaena* 7120 and *Nostoc* 29133 were pooled separately and washed twice with fresh $BG11_0$-medium. Such cultures were inoculated separately in 30 ml of fresh BG11 and $BG11_0$ media at equal chlorophyll *a* (Chl *a*) concentration, and MV was added to achieve final concentrations ranging from 0-0.4 µM, and incubated as described above. Growth was monitored periodically by measuring the concentration of Chl *a* in cyanobacterial cultures. Chl *a* was determined in the methanolic extracts by measuring absorbance at 663 nm×13.43, where 13.43 represents extinction coefficient of Chl *a* (Mackinney 1941).

5.2.3 Light microscopy: The cultures of *Anabaena* 7120 were examined under a light microscope (Olympus, Japan) at 400×magnification to determine the frequency of heterocysts, calculated as: Heterocyst frequency (%) = Number of heterocysts/Number of vegetative cells+Number of heterocysts×100.

5.2.4 Determination of total intracellular peroxides: The total peroxide levels was measured in the cultures of *Anabaena* 7120 after incubation in the presence of different concentrations of MV (0-0.4 µM) for two days by a ferrous oxidation/xylenol orange (FOX) assay method as described earlier (Wolff 1994; DeLong et al. 2002; Moirangthem et al. 2014).

5.2.5 In-gel determination of catalase activity: The cultures of *Anabaena* 7120 were exposed to MV for two days. Such cells were centrifuged at 2300×*g* for 5 min, and the cell pellets obtained were washed twice with 36 mM potassium phosphate buffer (pH 7.4). The cell-free extracts were obtained by sonication of the cell pellets in the same buffer added with 1 mM protease inhibitor phenylmethylsulphonyl fluoride (PMSF), as described earlier (Moirangthem et al. 2014). The total protein in cell extracts was measured according to Bradford (1976). Equal amounts of total protein from samples were separated on 12 % non-denaturing polyacrylamide gels (Laemmli 1970), following which the gels were stained for catalase activity according to Weydert and Cullen 2010, and visualized as achromatic bands on a greenish-yellow gel background.

5.2.6 Statistical analysis: All experiments were performed at least thrice and results are presented as mean ± standard deviation. The significant differences (*P* values less than 0.05) were analyzed by Student's two-sample *t*-test (Microsoft Excels version 10) between MV treated cyanobacterial cultures grown with and without nitrate and their respective controls.

5.3 Results and Discussion

5.3.1 MV tolerance in *Anabaena* 7120: *Anabaena* 7120 is highly versatile with regard to its ability to assimilate various forms of nitrogen as nutrients, including N_2 and nitrate (Bhattacharya et al. 2002; Frias and Flores 2015). *Anabaena* 7120 was cultivated in the presence of 17.6 mM nitrate (BG11-medium) and in absence of nitrate ($BG11_0$-medium; N_2-medium) to assess tolerance to MV (0, 0.2 and 0.4 µM). *Anabaena* 7120 grew well with and without nitrate (Fig. 5.1A and B, respectively) in the absence of MV, as judged by increase in Chl *a* content of cultures. When incubated with 0.2 µM MV, growth of *Anabaena* 7120 was inhibited by 68 % in the absence and by 30 % in the presence of nitrate, compared with respective untreated controls (100 %) after the experimental period of 6 days. Treatment with an increased dose of MV (0.4 µM) completely abolished growth of *Anabaena* 7120 in the absence of nitrate. However, when a similar dose of MV was applied to cultures growing with nitrate, growth was only inhibited by 60 % after 6 days. These results suggest that although MV is detrimental to growth of *Anabaena* 7120 both in the presence and absence of nitrate, sensitivity to this herbicide is considerably reduced in the presence of nitrate. The influence of nitrogen sources on MV sensitivity have been investigated by Raghavan et al. (2011, 2015) in *Anabaena* 7120, which is in contrast to the findings presented in this study, as these authors suggested equal sensitivity of this cyanobacterium to MV irrespective of whether it is grown with or without nitrate. To determine if nitrate is assimilated during MV stress, heterocyst frequency was determined in filaments of *Anabaena* 7120.

5.3.2 Impact of MV on heterocyst frequency in *Anabaena* 7120: Heterocysts, the N_2-fixing cells in filaments of *Anabaena* 7120 contain the enzyme nitrogenase (converts N_2 into ammonia) and are formed in the absence of fixed-nitrogen sources (Frias and Flores 2015). Both heterocyst formation and N_2-fixation are repressed in the presence of nitrate, and therefore, lack of heterocysts in filaments may serve as a good indicator of nitrate assimilation in *Anabaena* 7120. Thus, heterocyst frequency was determined in *Anabaena* 7120 (Fig. 5.2). Similar to control cultures grown without MV for 2 days, heterocyst frequency was repressed in 0.2 and 0.4 µM MV-treated cultures grown with nitrate. This repressive effect on heterocyst frequency was not detected in cultures treated with 0.2 µM MV in the absence of nitrate, as heterocyst frequency (approximately 4 %) was similar to untreated control. In cultures treated with 0.4 µM MV, no heterocysts could be detected, as filaments were massively fragmented. A lack of heterocysts indicates that such cultures may also lack nitrogenase. A loss of nitrogenase protein and activity has been demonstrated previously in MV-exposed *Nostoc punctiforme* ATCC 29133 (Moirangthem et al. 2014). Overall, the observations suggest that unlike N_2 assimilation, MV-treated cultures of *Anabaena* 7120 can assimilate nitrate. The nitrate assimilation pathway may not only help meet cellular nitrogen demand during MV stress, but also enable diversion of electrons generated by photosynthetic electron transport activity away from MV towards formation of ammonium (through nitrate reduction), resulting in reduced electron flow to MV and associated ROS formation. To evaluate the latter possibility, intracellular ROS level (measured as total hydroperoxide content) was determined.

5.3.3 Effect of MV on total hydroperoxide level: The intracellular hydroperoxide level was measured in cultures of *Anabaena* 7120 grown in the absence and in the presence of

nitrate with different concentrations of MV. As shown in Fig. 5.3, the control cultures of *Anabaena* 7120 grown in the absence of MV exhibited similar levels of intracellular hydroperoxides in the absence and in the presence of nitrate. In contrast, the total intracellular hydroperoxide levels increased in cultures exposed to MV, however, to varying degrees. With respect to untreated controls, the cultures incubated in the absence of nitrate with 0.2 and 0.4 µM MV showed a 2.14-fold and 5-fold increase, whereas similarly treated cultures in the presence of nitrate showed a 1.3-fold and 1.5-fold increase, respectively. These results suggest that growth on nitrate enables *Anabaena* 7120 to tightly control MV-induced hydroperoxide formation, as compared to when it is grown in the absence of nitrate. This is likely to be an effect of nitrate assimilation pathway acting as an electron sink thereby reducing electron flow toward MV reduction and consequent decreased formation of toxic ROS $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$ in nitrate grown cells, as opposed to that in cells grown in the absence of nitrate (Klotz et al. 2015). Since a lower intracellular hydroperoxide level may also be associated with increased H_2O_2 decomposition, catalase activity was assessed in the cultures of *Anabaena* 7120.

5.3.4 Effect of MV on catalase activity in *Anabaena* 7120: *Anabaena* 7120 possesses two Mn-containing catalases (Bernroitner et al. 2009). The catalase activity was evaluated in-gel in cultures of this cyanobacterium grown for 2 days in the presence and absence of nitrate and MV. As shown in Fig. 5.4, *Anabaena* 7120 grown in the absence of nitrate exhibited a single catalase activity band, the intensity of which increased in 0.4 µM MV treated cultures. This increased catalase activity is likely to be an adaptive response of cells to decompose and detoxify high concentrations of intracellular hydroperoxide generated by MV (Latifi et al. 2009; Moirangthem et al. 2014). On the other hand, cultures

grown in the presence of nitrate with and without MV did not exhibit any catalase activity band. These results indicate that the lower intracellular hydroperoxide levels observed in response to MV in nitrate grown cells was not due to catalase mediated decomposition of H_2O_2. Although one cannot exclude the involvement of Prxs in reducing H_2O_2 levels, transcriptional studies suggest that the expression of Prxs is generally higher in cells grown without nitrate than in cells grown with nitrate (Cha et al. 2007). Taken together, the results indicate a role of nitrate assimilation pathway in reducing intracellular hydroperoxide levels thereby improving MV tolerance in *Anabaena* 7120. Further, to determine if this nitrate-mediated protective phenomenon also holds true for other filamentous cyanobacteria, MV sensitivity of *Nostoc* 29133 was evaluated in media supplemented with and without nitrate.

5.3.5 MV tolerance in *Nostoc* 29133: To assess MV tolerance, *Nostoc* 29133 was cultivated in the presence of 17.6 mM nitrate (BG11-medium) (Fig. 5.5A) and in absence of nitrate ($BG11_0$-medium; N_2-medium) (Fig. 5.5B) with and without MV (0.2 and 0.4 µM) as described for *Anabaena* 7120. As shown in Fig. 5.5A and B, *Nostoc* 29133 showed reduced MV sensitivity when grown in the presence of nitrate than in its absence, as was observed for *Anabaena* 7120, suggesting that nitrate-mediated protection against MV may be a conserved feature in filamentous cyanobacteria.

5.3.6 Conclusion: The present study established that filamentous cyanobacteria *Anabaena* 7120 and *Nostoc* 29133 exhibit improved tolerance to oxidative stress imposed by MV, a redox-cycling ROS-generating herbicide, when grown in the presence of nitrate than when grown in its absence. As compared to MV-stressed *Anabaena* 7120 grown

without nitrate, the nitrate grown cultures generated lower amount of hydroperoxides. This reduction in hydroperoxide level was independent of catalase activity. It was concluded that the protective effect of nitrate may be linked to its assimilation mechanism (nitrate and nitrite reduction), which consumes photosynthetically generated electrons (eight per nitrogen atom fixed), and thereby reduce electron flow to MV leading to reduced production of ROS and lower oxidative stress. Further studies using nitrate-assimilation defective mutants may provide stronger evidence for the role of nitrate in modulating MV tolerance in filamentous cyanobacteria. This may have implications for growth and survival of these important microorganisms in agricultural fields, which are often loaded with MV and nitrate fertilizers.

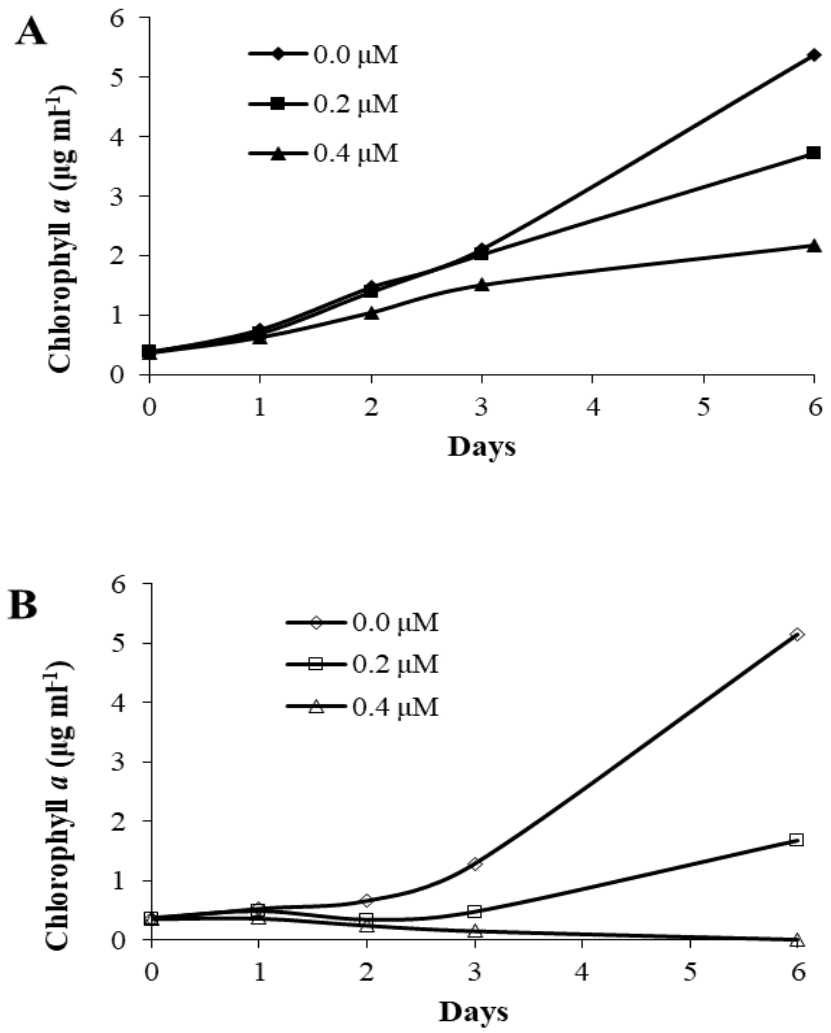

Fig. 5.1 Effect of MV on growth of *Anabaena* 7120 in the presence (A) and in the absence (B) of nitrate. Exponentially growing cultures of *Anabaena* 7120 were harvested and washed twice with fresh BG11o-medium (lacking nitrate), and such cultures were transferred at equal density to fresh medium with and without nitrate and added with increasing concentrations of MV. Growth was measured at regular intervals by determining Chl *a* content. Depicted error bars mostly smaller than the symbols, represent standard deviation of two experiments with independent cultures.

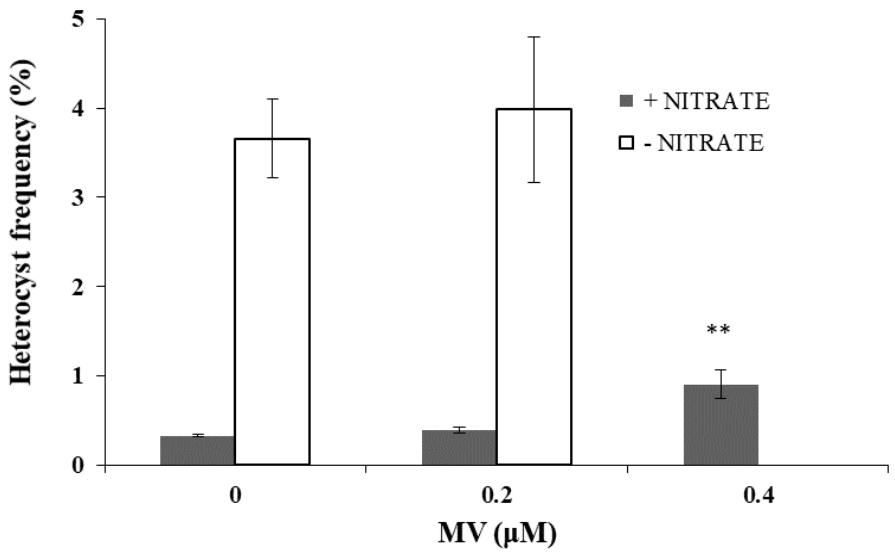

Fig. 5.2 Effect of MV on heterocyst frequency of *Anabaena* 7120 in the presence and in the absence of nitrate. Error bars shown represent standard deviation of two independent experiments. Asterisks (**, $P<0.01$) above the bar denote significant difference from control values

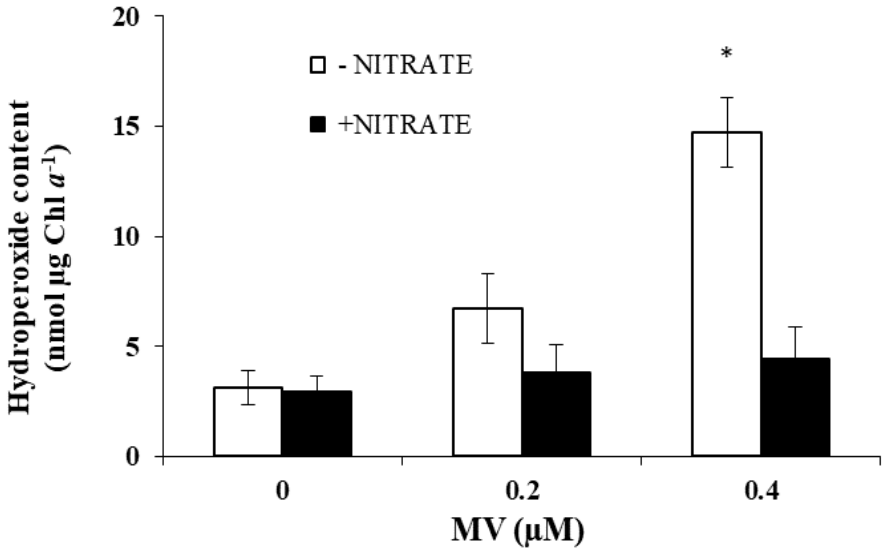

Fig. 5.3 Effect of MV on total hydroperoxide levels in *Anabaena* 7120. Exponentially growing cultures of *Anabaena* 7120 were incubated at equal Chl *a* density (0.5 µg ml^{-1}) for 2 days with and without nitrate and MV in fresh medium. Such cultures were washed twice with 36 mM phosphate buffer (pH 7.8) followed by measurement of total hydroperoxide levels in cell pellets by ferrous iron oxidation-xylenol orange (FOX) assay. Depicted error bars represent standard deviation of three independent experiments

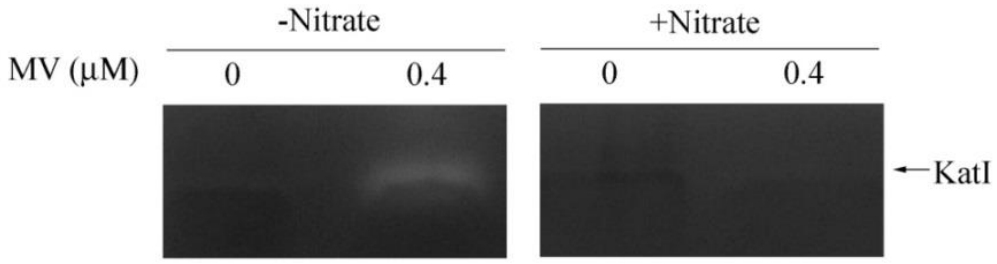

Fig. 5.4 Effect of MV on catalase activity in *Anabaena* 7120. The cyanobacterial cultures treated or untreated with 0.4 µM MV for 2 days in the presence and absence of nitrate were harvested, washed and resuspended in 36 mM phosphate buffer (pH 7.4) and broken by ultrasonication. The cell extracts equivalent to 20 µg total protein were separated by native PAGE (12 % gel) and stained for catalase activity. Similar results were obtained from two independent experiments, and a representative gel is shown

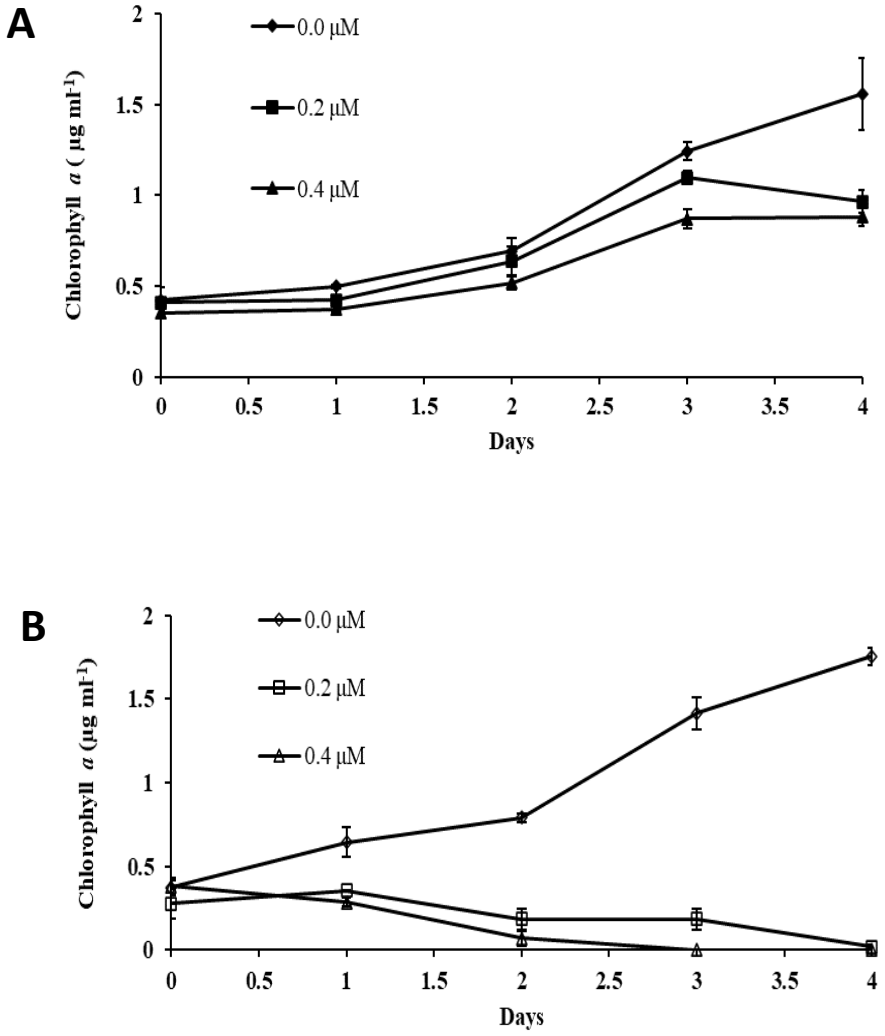

Fig. 5.5 Effect of MV on growth of *Nostoc* 29133 in the presence (A) and in the absence (B) of nitrate. Exponentially growing cultures of *Nostoc punctiforme* were harvested and washed twice with fresh BG11o-medium (lacking nitrate), and such cultures were transferred at equal density to fresh medium with and without nitrate and added with increasing concentrations of MV. Growth was measured at regular intervals by determining Chl *a* content. Depicted error bars sometimes smaller than the symbols, represent standard deviation of two experiments with independent cultures.

CHAPTER 6

Summary

Being photoautotrophic in nature, cyanobacteria must confront a variety of reactive oxygen species (ROS) generated from photosynthesis and/ or respiratory electron transport pathways. Sub-optimal growth conditions often encountered in natural environments increase the production of ROS in cyanobacteria. Excess ROS is highly detrimental to growth and survival of these ecologically, agriculturally, and biotechnologically important microorganisms. The present study embodies characterization of the physiological and biochemical responses of filamentous diazotrophic cyanobacteria *Nostoc punctiforme* strain ATCC 29133-S (*Nostoc* 29133) and *Anabaena* sp. PCC 7120 (*Anabaena* 7120) to oxidative stress induced by exogenous application of inorganic (H_2O_2) and organic hydroperoxides (*tert*-butyl hydroperoxide; *t*-BOOH), and methyl viologen (MV), a redox-cycling ROS-generating herbicide. The findings of this study are summarized below:

1. H_2O_2 tolerance ability of *Nostoc* 29133 was evaluated and compared with respect to *Anabaena* 7120. The results demonstrated that *Nostoc* 29133 was better able to tolerate H_2O_2-induced inhibition of chlorophyll *a* and photosystem II performance, as compared to *Anabaena* 7120. The intracellular hydroperoxide level (indicator of oxidative status) also did not exhibit as much a rise in *Nostoc* 29133, as it did in *Anabaena* 7120 after H_2O_2 treatment. Accordingly, *Nostoc* 29133 showed higher intrinsic constitutive catalase activity than *Anabaena* 7120 indicating that the superior tolerance of *Nostoc* 29133 stems from its higher ability to decompose H_2O_2, suggesting that even closely related filamentous cyanobacteria may differ in terms of H_2O_2 tolerance. H_2O_2 stress tolerant photoautotrophic cyanobacterium like *Nostoc* 29133 is likely to be an important

biotechnological resource, and may be exploited as a potential source of valuable antioxidant catalase.

2. To determine if *Nostoc* 29133 and *Anabaena* 7120 also vary in their ability to tolerate organic hydroperoxide, the growth response of these two cyanobacteria to *t*-BOOH, an analog of organic hydroperoxide, was examined. The results indicated that *Nostoc* 29133 and *Anabaena* 7120 are equally sensitive to *t*-BOOH, unlike H_2O_2. Further characterization of *Nostoc* 29133 revealed that increased accumulation of intracellular hydroperoxides resulting from inability of this cyanobacterium to quickly and completely detoxify *t*-BOOH, presumably via activity of peroxiredoxins, was the basis for its sensitivity to *t*-BOOH. The activity of antioxidative enzyme superoxide dismutase was not influenced by *t*-BOOH, but a new catalase isoenzyme was induced in response to this oxidant in *Nostoc* 29133. These findings suggest that in addition to *t*-BOOH detoxifying peroxiredoxins, catalase activity may also be important to tackle *t*-BOOH induced oxidative stress in *Nostoc* 29133. Further studies using knockout mutants may be necessary to determine the precise roles of each of these enzymatic antioxidants in mitigating organic hydroperoxide stress in cyanobacteria.

3. An investigation on the responses of *Anabaena* 7120 and *Nostoc* 29133 to MV (an endogenous producer of peroxides) revealed that these cyanobacteria can tolerate MV stress better when grown in the presence of nitrate (a fixed-nitrogen nutrient) than in its absence (diazotrophic growth). The MV-stressed nitrate grown *Anabaena* 7120 generated lower amount of hydroperoxides, as compared to when it is grown diazotrophically. This reduction in hydroperoxide level was independent of catalase activity. It was concluded

that the protective effect of nitrate may be linked to its assimilation mechanism (nitrate and nitrite reduction), which consumes photosynthetically generated electrons (eight per nitrogen atom fixed), and thereby reduce electron flow to MV leading to reduced production of ROS and lower oxidative stress. Further studies using nitrate-assimilation defective mutants may provide stronger evidence for the role of nitrate in modulating MV tolerance in filamentous cyanobacteria. This may have implications for growth and survival of these important microorganisms in agricultural fields, which are often loaded with MV and nitrate fertilizers.

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The 2,2' Dipyridyl-Induced Iron Starvation and its Effects on Growth and Photosynthesis in Cyanobacterium *Nostoc punctiforme* ATCC 29133

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Abstract—Iron is essential for growth of most organisms, including cyanobacteria, a ubiquitous and ecologically important group of microorganisms in nature. The present study was initiated to investigate the effects of iron starvation on the growth, frequency of heterocysts (the sites for nitrogen-fixation), photosynthetic pigments and photosynthesis in the filamentous, nitrogen-fixing cyanobacterium *Nostoc punctiforme* ATCC 29133. Iron starvation was achieved in cyanobacterial cultures by growing them in medium free of combined nitrogen containing 2,2'dipyridyl (a high affinity iron-chelator) without any addition of iron source. Compared to iron-sufficient control cultures, the iron-starved cultures showed decrease in growth determined for 15 days. The reduction in growth was coupled with a decreased heterocyst (N_2-fixation sites) frequency and the number of cells per filament measured after 2 and 4 days of iron-starvation. Similarly, a considerable drop in the concentration of photosynthetic pigments such as, phycocyanin and chlorophyll *a* were also noticed in iron-starved cultures. Carotenoid level, however, was higher in iron-starved cultures compared to control. The maximum quantum efficiency of photosystem II photochemistry indicating the photosynthetic efficiency was severely affected in iron-starved *Nostoc punctiforme* ATCC 29133. Overall, the results presented in this study suggest that deficiency of iron negatively impacts growth, photosynthesis and perhaps nitrogen-fixation in the cyanobacterium *N. punctiforme* ATCC 29133. Given the role of cyanobacteria in biofertilizer technology, it is suggested that iron bio-availability in agricultural fields may strongly impact the biofertilizer potential of diazotrophic cyanobacteria. Therefore, efforts to improve biofertilizer potential of cyanobacteria may be directed towards identifying strains which can better adapt to iron deficiency.

Keywords: Cyanobacteria, *Nostoc punctiforme*, Iron starvation, Heterocyst, Photosynthetic pigments.

INTRODUCTION

Iron is one of the most abundant elements on earth's crust (5%). It exists in two redox forms, ferrous (Fe^{2+}) and ferric (Fe^{3+}). The Fe^{2+} (soluble) is rapidly oxidized to Fe^{3+} in aerobic and alkaline environments, leading to predominance of Fe^{3+} (insoluble) in many ecosystems ranging from earth's oceans to soils. Thus, poor availability of iron, an essential nutritional element, becomes a key issue for most organisms, including oxygen-evolving photosynthetic cyanobacteria (Straus, 1994; Michel and Pistorius, 2004). Cyanobacteria exhibit many alterations in morphology, physiology, and gene expression as a response to iron deficiency. Prominent among them are changes in cell size, filament length reduction, loss of phycobilins, components of light harvesting pigment-protein complexes. The photosynthetic apparatus composed of photosystem II (PSII), PSI, the intersystem electron transport complex cytochrome b_6f, and the terminal electron acceptor ferredoxin are also synthesized at reduced levels in cyanobacteria undergoing iron starvation due to high requirement of iron (approximately 23-24 atoms) (Shi *et al*., 2007). As a consequence the photosynthetic activity is severely reduced in iron-starved cyanobacteria. Similarly, in diazotrophic cyanobacteria, the frequency of heterocysts, (sites of N_2-fixation) transcript levels of nitrogenase-encoding genes (*nifHDK*), and nitrogenase activity have all been found to decrease considerably in diazotrophs facing iron limitation (Narayan *et al*., 2011; Shi *et al*., 2007; Wen-

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Liang *et al*., 2003). The assimilation of nitrate and nitrite (alternate nitrogen sources) also seems to be affected as nitrate- and nitrite-reductases lose their activities due to shortage of electron-donor, ferredoxin (Hardie *et al*., 1983; Verstreate *et al*., 1980). Thus, deficiency of iron creates secondary nitrogen limitation in cyanobacteria (Straus, 1994). Indeed, low iron availability has been reported to limit growth of phytoplanktons in many parts of the world's oceans (Behrenfeld *et al*., 1996).

In many cyanobacteria, adaptation to iron deficiency includes synthesis and secretion of siderophores and induction of highly efficient iron acquisition systems to scavenge iron from the environment (Hopkinson and Morel, 2009; Michel and Pistorius, 2004; Straus, 1994). Iron thus acquired from the environment is then stored in ferritin-like proteins to be used during protracted periods of iron starvation (Ekman *et al*., 2014). The accumulation of an alternate Chlorophyll (Chl) *a*-binding complex CP43', encoded by *isiA* (iron stress induced protein A), has also been shown to be important for acclimation to iron deficiency in many cyanobacteria (Michel and Pistorius, 2004; Singh and Sherman, 2007). IsiA binds to PSI trimers forming an IsiA-PSI supercomplex that increases effective cross-section of PSI so that more light can be harvested to sustain photosynthesis in absence of iron. Such large aggregates of IsiA can also dissipate excess light energy and thus protect cells from oxidative stress whether associated or not with PSI (Bibby *et al*., 2001; Boekema *et al*., 2001; Wang *et al*., 2010). It has also been proposed that IsiA (Chl-reserve) may assist cells recover faster from iron deficiency stress by providing Chl to PSI, if and when iron becomes available (Fraser *et al*., 2013; Singh and Sherman, 2007). Additionally, many cyanobacteria synthesize flavodoxin to functionally compensate the loss of iron-rich ferredoxin during iron-deficient growth (Michel and Pistorius, 2004; Straus, 1994).

A bioinformatics-based analysis has revealed that the filamentous, N_2-fixing cyanobacterium *Nostoc punctiforme* do not possess the gene encoding for *isiA* in its genome (Singh *et al*., 2004). Similarly, it lacks the genes encoding for citrate-based siderophores (Hopkinson and Morel, 2009). Despite these unusual features, its response to iron starvation has not been studied earlier. Therefore, the present study was undertaken to determine the impact of iron starvation on growth, cellular morphology, pigmentation, and PSII photochemistry of this cyanobacterium.

MATERIALS AND METHODS

Strain and Culture Conditions: The cyanobacterium *Nostoc punctiforme* (hereafter referred to as *N. punctiforme*) was grown photoautotrophically at 25 °C in $BG11_0$-medium (pH 7.5) containing 30 µM ferric ammonium citrate (+Fe medium), and $BG11_0$-medium lacking ferric ammonium citrate, but added with 50 µM 2,2'-dipyridyl (-Fe medium) under continuous illumination (20 µmol m^{-2} s^{-1}) (Rippka *et al*., 1979).

Microscopy: The number of cells was counted in approximately 7-10 filaments (vegetative cells and heterocysts) of *N. punctiforme* culture using a light microscope (Olympus). The frequency of heterocysts was calculated as: Heterocyst frequency (%) = Number of heterocysts/Number of vegetative cells + Number of heterocysts ×100.

Measurement of Growth: The growth of *N. punctiforme* cultures was measured by following optical density of cell suspensions at 750 nm, in a Cary 60 spectrophotometer (Agilent Technologies, USA).

Measurements of Photosynthetic Pigments: The levels of photosynthetic pigments phycocyanin (PC), chlorophyll *a* (Chl *a*), and carotenoids (Car) of intact *N. punctiforme* cultures were determined from the absorption spectra (visible range; 400-800nm) obtained using a Cary 60 spectrophotometer. The concentrations of PC were calculated from absorption at 620nm, Chl *a* at 680nm, and Car at 490 nm. The values of PC/cell, Chl *a*/cell, and Car/cell were derived using the formula $(A_{620}-A_{750})/A_{750}$, $(A_{680}-A_{750})/A_{750}$, and $(A_{495}-A_{750})/A_{750}$, respectively (Fraser *et al*., 2013).

Photosynthetic Measurement by Chlorophyll Fluorescence: The chlorophyll fluorescence is a widely used in vivo method to measure photosynthetic performance of plants, algae and cyanobacteria (Baker, 2008). A pulse amplitude modulated fluorometer, Dual-PAM-100, (Waltz, Effeltrich, Germany) was used for photosynthetic measurements in cultures of *Nostoc punctiforme*, stirred in 1 cm^{-1} cm cuvettes at 25 °C. The maximum quantum efficiency of photosystem II (PSII) photochemistry (F_v/F_m; reflects maximum efficiency at which light absorbed by PSII is used for reduction of primary quinine electron acceptor of PSII, Q_A) was determined after incubation of the cyanobacterial cultures in dark for 15 min followed by illumination of the sample with a low-irradiance measuring light (0.24 µmol m^{-2} s^{-1}) to determine F_o (minimal fluorescence yield of dark-adapted samples with all PSII centers open/oxidized). A saturating pulse of white light (600 ms, 1000 µmol m^{-2} s^{-1}) was then applied to the sample to determine F_m (maximal fluorescence of dark-adapted samples with all PSII centers closed/reduced). The F_v value (variable fluorescence of dark-adapted samples) was then derived from (F_m-F_0) (Genty *et al*., 1989).

RESULTS

The effect of iron-limitation on growth was assessed by using log-phase cultures of *N. punctiforme* grown in iron-sufficient BG11o-medium as source of inoculum. Such cultures were washed twice with $BG11_0$-medium devoid of any iron source (30 μM of ferric ammonium citrate) before re-suspending in the same medium containing 50 μM of 2,2'-dipyridyl, an high affinity iron chelator (-Fe-medium). The changes in optical density (OD) of cultures were periodically monitored at 750 nm. As shown in Fig. 1, *N. punctiforme* grew well in medium containing normal concentration of iron. However, its growth was completely ceased in medium devoid of free iron. The number of cells per filament and frequency of heterocysts were evaluated by light microscopy in cultures of *N. punctiforme* after 2 and 4 days of incubation in medium with and without iron. Such examination did not reveal lysis of cells. Compared to iron-sufficient cultures, the number of cells per filament in *N. punctiforme* (Fig. 2 A) dropped from 67 to 38 and from 76 to 26 after 2 and 4 days of incubation, respectively. Whereas, an approximately 50 % drop was noted in the frequency of heterocysts in iron-starved cultures after day 2. No further significant drop in heterocyst frequency was detected after day 4 (Fig. 2B). These results suggest that iron starvation leads not only to reduction in growth, but also reduces number of cells per filament and frequency of heterocysts in *N. punctiforme*.

Such effects on growth and morphology of iron-starved *N. punctiforme* was paralleled by a clear change in cellular pigment concentration; the appearance of *N. punctiforme* cultures changed progressively from blue green to yellow as incubation was prolonged in medium devoid of iron (data not shown). To quantify the changes in the concentration of photosynthetic pigments, chlorophyll *a* (Chl *a*), phycocyanin (PC), and carotenoid (Car) levels were determined from the whole-cell absorption spectra of iron-sufficient and iron-starved cultures of *N. punctiforme* (Fig. 3 A, B and C). The levels of both Chl *a* and PC decreased in iron-starved cultures compared with their respective iron-sufficient cultures on per cell basis. The reduction in Chl *a* concentration was faster than reduction in PC level. However, the Car levels per cell did not decrease in the iron-starved cultures, and instead, was maintained at somewhat higher levels than in iron-sufficient cultures. These results suggest that, except Car, iron starvation impacts the accumulation of Chl *a* and PC in *N. punctiforme*. The photosynthetic performance was also measured in the cultures of *N. punctiforme* iron-starved for 2 days. The maximal PSII quantum efficiency (F_v/F_m) in iron-starved *N. punctiforme* (Fig. 4) decreased by 58 %, compared with control cultures. These results suggested that iron starvation affects photosynthetic pigments and photosynthetic activity in *N. punctiforme*.

Fig. 1. Effect of iron starvation on growth of *N. punctiforme.* The log-phase cultures of *N. punctiforme* were harvested and washed. Such cultures were transferred to $BG11_0$-medium (iron-sufficient, +Fe; iron-deficient, containing 50 μM 2,2'-dipyridyl and lacking ferric ammonium citrate, -Fe) at equal culture density and growth was measured at regular intervals by following optical density (OD) at 750 nm. Error bars, sometimes smaller than the symbols, represent mean±standard deviation of three experiments.

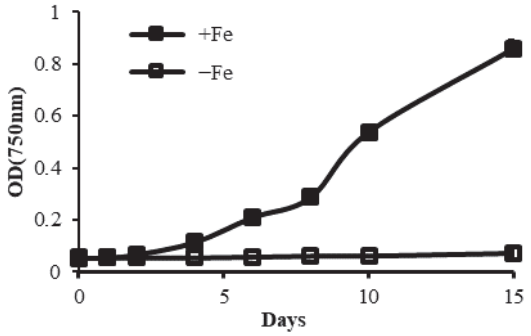

Fig. 1

Fig. 2. Effect of iron starvation on number of cells per filament and heterocyst frequency in *N. punctiforme*. The number of cells per filament and heterocyst frequency (A and B, respectively) was counted in 2 and 4 days-old +Fe and -Fe grown cultures. Approximately 1000 cells of each were counted under a light microscope and the error bars shown represent mean±standard deviation of three experiments. Asterisks (**, P<0.01; ***, P<0.001) above the bar denote significant difference from control values.

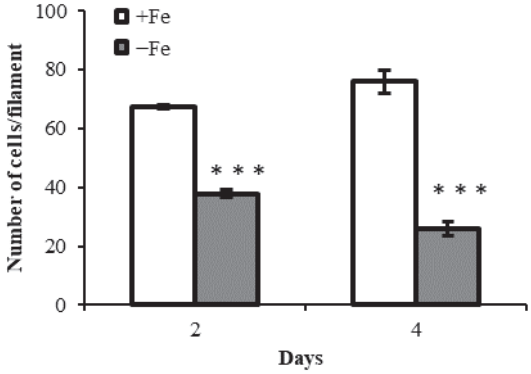

Fig. 2A

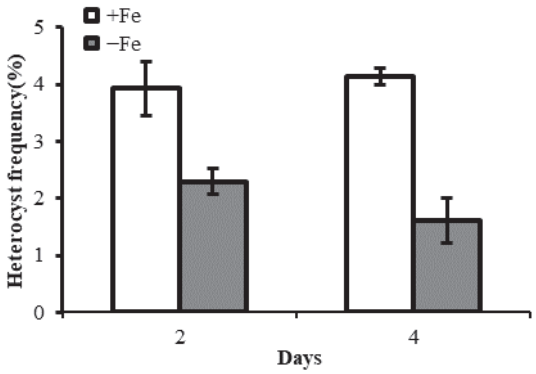

Fig. 2B

Fig. 3. Effect of iron starvation on photosynthetic pigments in *N. punctiforme*. The pigment levels, chlorophyll *a* (Chl *a*/cell) phycocyanin (PC/cell) and carotenoid (Car/cell) (A, B and C, respectively) were determined from whole-cell absorption spectra of 2, 4, and 8-days old +Fe and –Fe grown cultures. Error bars shown represent mean±standard deviation of three experiments.

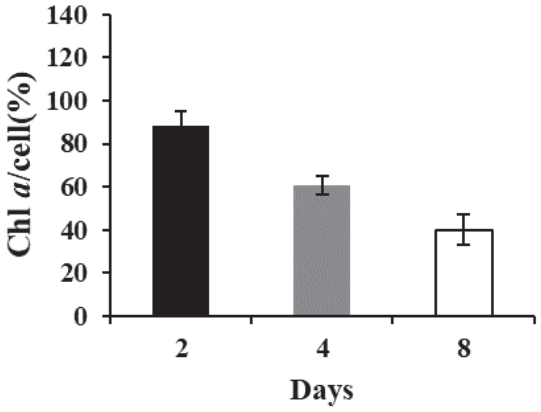

Fig. 3A

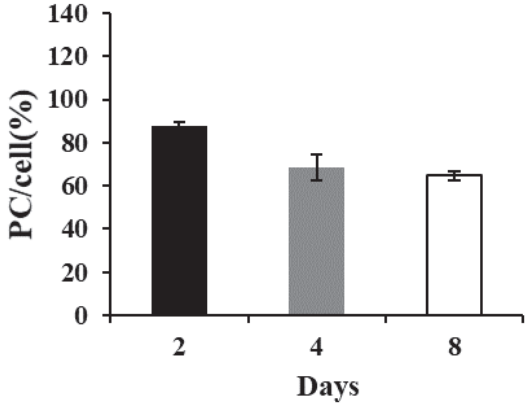

Fig. 3B

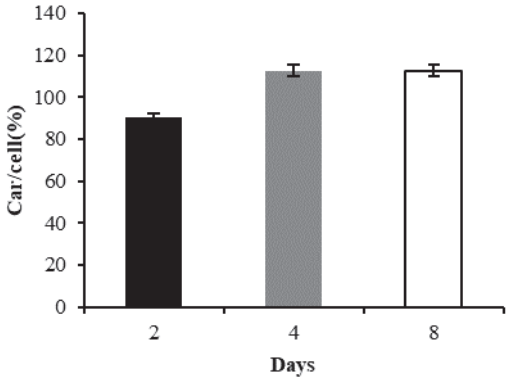

Fig. 3C

Fig. 4. Effect of iron starvation on maximal quantum efficiency (F_v/F_m) of PSII photochemistry in *N. punctiforme*. The 2-days old cultures grown in $BG11_0$-medium (iron-sufficient, +Fe; iron-deficient, containing 50 µM 2,2'-dipyridyl and lacking ferric ammonium citrate, -Fe) were harvested, washed and used for maximal quantum efficiency measurement at a Chl *a* concentration of 3 µg Chl *a*.ml^{-1} by pulse amplitude modulated fluorometry. Error bars represent mean±standard deviation of three experiments.

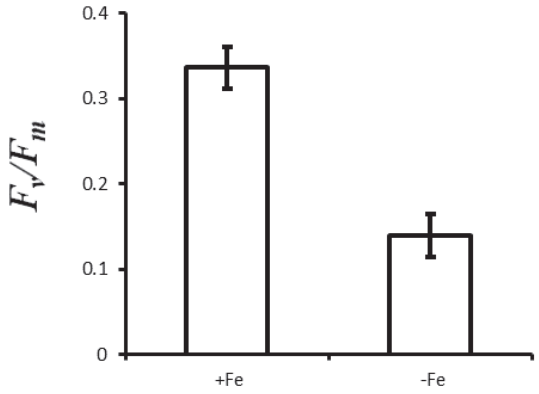

Fig. 4

DISCUSSION

The method commonly used to hasten the process of iron-starvation in cyanobacteria is to add iron-chelators (deferoxamine B/diethylenetriaminopentaacetic acid/EDTA) to iron-free growth medium (Shcolnick *et al*., 2009). During the present study, quick and complete growth arrest was achieved in *N. punctiforme* by the addition of 2,2'-dipyridyl (50 µM) in $BG11_0$-medium lacking iron-source. Similar effect of 2,2'-dipyridyl, an high affinity iron chelator, has been observed earlier on growth of *Anabaena* PCC 7120 and *Synechocystis* PCC 6803 (Cheng and He,

2014; Latifi *et al*., 2005; Xu *et al*., 2003). Morphological analysis revealed that the number of cells per filament of *N. punctiforme* reduced as the incubation continued in iron-free medium. A similar fragmentation and short filaments were also noticed in *Trichodesmium* IMS101 during iron-limited growth (Küpper *et al*., 2008). In *Synechococcus elongatus*, the cell length was found to be reduced, whereas enlarged and vacuolated cells were observed in filaments of *Fremyella diplosiphon* in response to iron-limitation (Benešová *et al*., 2000; Pattanaik *et al*., 2014). These results suggest that iron starvation differentially affects cellular morphology in cyanobacteria. Concomitant with changes in number of cells per filament, the frequency of heterocysts also declined in iron-starved cultures. Since nitrogenase (catalyzes conversion of molecular nitrogen into ammonia) is localized in heterocysts, it is quite possible that a loss in heterocyst frequency would also be paralleled by simultaneous decrease in nitrogenase abundance and activity. Indeed, iron-starvation has been shown to affect heterocyst frequency, transcript levels of nitrogenase and activity in diazotrophic cyanobacteria (Narayan *et al*., 2011; Shi *et al*., 2007; Wen-Liang *et al*., 2003).

One of the visual indicators of iron-deficient growth in cyanobacteria is chlorosis, also observed in iron-starved *N. punctiforme*. It is known that the biosynthesis of chromophores requires haem and ferredoxin-dependent reductases (Beale, 1994). Hence, it is quite likely that lack of iron would lead to decreased synthesis of photosynthetic pigments (Cheng and He, 2014; Fraser *et al*., 2013; Michel and Pistorius, 2004; Straus, 1994). Consistent with this reasoning, pigment analysis revealed that the accumulation of PC and Chl *a* reduced in iron-starved cultures this cyanobacterium compared with its iron-sufficient cultures. It has been demonstrated earlier that there are variations among cyanobacteria in the way they modulate their photosynthetic pigment levels during iron-limited growth; the PC/Chl *a* ratio decreases in *Synechococcus elongatus* and *Thermosynechococcus elongatus*, whereas this ratio either increases or remains constant in *Synechocystis* PCC 6803 and *Arthrospira maxima* (Boulay *et al*., 2008). Possibly, *N. punctiforme* represents the latter group, as its PC/Chl *a* ratio was slightly higher during iron-starved growth than when it was grown with sufficient iron. The pigment analysis also suggested that the Car/cell levels increased in iron-starved cultures of *N. punctiforme* compared to control cultures. Possibly, enhanced carotenoid levels dissipate excess light energy, and thereby curtail iron starvation induced formation of reactive oxygen species (ROS) in *N. punctiforme* (Boulay *et al*., 2008). An increase in carotenoid level has been detected earlier in an IsiA-lacking mutant of *Synechococcus* PCC 7942 (Burnap *et al*., 1993). Furthermore, the iron-starved *N. punctiforme* cultures displayed marked alteration in PSII photochemistry. The maximum quantum efficiency of open PSII centers measured as F_v/F_m ratio decreased considerably in iron-starved cultures, suggesting that the photosynthetic performance of this cyanobacterium is severely compromised due to lack of iron. Such an effect of iron-starvation on photosynthetic performance has also been demonstrated for many other iron-starved cyanobacteria (Cheng and He, 2014; Fraser *et al*., 2013; Ivanov *et al*., 2000; Straus, 1994).

CONCLUSION

The results presented in this study suggests that 2,2'-dipyridyl induced iron starvation leads to multiple defects in the diazotrophic cyanobacterium *N. punctiforme*. Such defects include (1) impairment of diazotrophic growth, (2) reduction in number of cells per filament, (3) decrease in heterocyst frequency, (4) reduction in the levels of phycocyanin and chlorophyll *a*, but not carotenoids, and (5) decreased PSII photochemistry measured as maximal quantum efficiency of photosynthesis. Based on these observations in *N. punctiforme*, it is suggested that poor bio-availability of iron, commonly occurring in many agricultural fields, may be a crucial factor limiting biofertilizer potential of diazotrophic cyanobacteria.

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ORIGINAL PAPER

Differential catalase activity and tolerance to hydrogen peroxide in the filamentous cyanobacteria *Nostoc punctiforme* ATCC 29133 and *Anabaena* sp. PCC 7120

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Abstract

Photoautotrophic cyanobacteria often confront hydrogen peroxide (H_2O_2), a reactive oxygen species potentially toxic to cells when present in sufficiently high concentrations. In this study, H_2O_2 tolerance ability of filamentous cyanobacteria *Nostoc punctiforme* ATCC 29133 (*Nostoc* 29133) and *Anabaena* sp. PCC 7120 (*Anabaena* 7120) was investigated at increasing concentrations of H_2O_2 (0–0.5 mM). In *Nostoc* 29133, 0.25 and 0.5 mM H_2O_2 caused a reduction in chlorophyll *a* content by 12 and 20%, respectively, whereas with similar treatments, a total loss of chlorophyll *a* was detected in *Anabaena* 7120. Further, *Nostoc* 29133 was able to maintain its photosystem II performance in the presence of H_2O_2 up to a concentration of 0.5 mM, whereas in *Anabaena* 7120, 0.25 mM H_2O_2 caused a complete reduction of photosystem II performance. The intracellular hydroperoxide level (indicator of oxidative status) did not increase to the same high level in *Nostoc* 29133, as compared to in *Anabaena* 7120 after H_2O_2 treatment. This might be explained by that *Nostoc* 29133 showed a 20-fold higher intrinsic constitutive catalase activity than *Anabaena* 7120, thus indicating that the superior tolerance of *Nostoc* 29133 to H_2O_2 stems from its higher ability to decompose H_2O_2. It is suggested that difference in H_2O_2 tolerance between closely related filamentous cyanobacteria, as revealed in this study, may be taken into account for judicious selection and effective use of strains in biotechnological applications.

Keywords *Anabaena* · Catalase · Cyanobacteria · Hydrogen peroxide · *Nostoc* · Oxidative stress

Introduction

Hydrogen peroxide (H_2O_2) is one of the several reactive oxygen species (ROS) produced as a by-product of photosynthesis and/or respiratory processes in aerobic organisms (Latifi et al. 2009; Imlay 2013). The oxygen-evolving photosynthetic cyanobacteria, major drivers of global carbon and nitrogen cycle, and potential sources of biofuels and commodity chemicals, produce H_2O_2 mainly through superoxide dismutase (SOD) catalyzed disproportionation of superoxide radical ($O_2^{\bullet-}$), a by-product of photosynthetic electron transport activity (Banerjee et al. 2013; Singh et al. 2016; Kitchener and Grunden 2018). Incomplete oxidation of H_2O at the donor side of photosystem II (PSII) also generates H_2O_2 in cyanobacteria (Pospíšil 2009). A wide variety of naturally occurring stressors, such as high light, ultraviolet rays, salinity, herbicides, heavy metals, and high and low temperature, further increase the intracellular concentration of H_2O_2 in cyanobacteria (Latifi et al. 2009; Chauvat and Chauvat 2015; Mironov et al. 2019). Besides, cyanobacteria may also encounter H_2O_2 sourced from metabolic activities of other organisms or from photo-oxidation of chromophoric dissolved organic matter in their natural environments (Diaz and Plummer 2018; Zinser 2018).

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While at low concentration, H_2O_2 may function as a second messenger in cell signal transduction pathways; sufficiently high concentrations of H_2O_2 cause oxidative stress leading to loss of membrane integrity, destruction of light-harvesting pigments, impairment of PSII reaction center protein D1 and photosynthetic activity, and ultimately cell death in cyanobacteria (Drábková et al. 2007a, b; Nishiyama et al. 2011; Mikula et al. 2012; Yang et al. 2018; Piel et al. 2019). It has been suggested that such adverse effects are most often not directly caused by H_2O_2, but rather by the ROS hydroxyl radical ($HO^{\bullet}$) formed from interaction of H_2O_2 with free intracellular ferrous iron via Fenton chemistry (Imlay 2013). Hence, cyanobacteria must promptly neutralize H_2O_2 to avoid formation of cell lethal $HO^{\bullet}$.

Cyanobacteria possess various antioxidative enzymes, such as thiol-specific peroxiredoxins (Prxs) and catalases to neutralize H_2O_2 (Latifi et al. 2009; Banerjee et al. 2013). Whereas Prxs reduce low concentrations of H_2O_2 (K_M in µM range), catalases efficiently decompose high concentrations of H_2O_2 (K_M in mM range) (Tichy and Vermaas 1999). Comparative genome sequence analysis in cyanobacteria has revealed that unlike Prxs, the distribution of catalases is not uniform; a large number of them (nearly 50%) lack catalase-encoding gene (Bernroitner et al. 2009). Accordingly, several studies also revealed higher sensitivity to H_2O_2 in some cyanobacteria which lack catalase, such as the unicellular fresh water *Microcystis aeruginosa* and marine-dwelling *Prochlorococcus* (Morris et al. 2011; Mikula et al. 2012). Conversely, the ones containing catalase (heme-dependent KatG)-like unicellular, non-diazotrophic cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 have been shown to tolerate high concentrations of H_2O_2 (Tichy and Vermaas 1999; Perelman et al. 2003). On the other hand, many KatG-lacking filamentous diazotrophic cyanobacteria, particularly the species of *Anabaena*, which possess at least one catalase with manganese as cofactor (Mn catalase), are reported to lack catalase activity, irrespective of whether they were grown in the presence or absence of H_2O_2 (Bagchi et al. 1991; Bernroitner et al. 2009; Banerjee et al. 2012; Chakravarty et al. 2016; Ballal et al. 2020). Correspondingly, *Anabaena* sp. PCC 7120, despite possessing two Mn catalases, was more sensitive to H_2O_2, as compared to *Synechocystis* sp. PCC 6803 (Pascual et al. 2010). A report also suggests that owing to higher basal level catalase activity *Synechococcus* sp. PCC 7942 is more tolerant to H_2O_2 than *Synechocystis* sp. PCC 6803 (Gupta and Ballal 2015). It is presently unclear whether variation in H_2O_2 tolerance also exists among filamentous cyanobacteria. Such information may be important, as this may lead to identification of stress resilient strains to be used in biotechnological applications (Kitchener and Grunden 2018).

In this study, H_2O_2 tolerance ability of *Nostoc punctiforme* ATCC 29133 (hereafter *Nostoc* 29133) was evaluated with respect to the reference strain *Anabaena* sp. PCC 7120 (hereafter *Anabaena* 7120) to determine possible differences, if any, between these two taxonomically closely related filamentous cyanobacteria. However, unlike *Anabaena* 7120, *Nostoc* 29133 has a symbiotic origin and was isolated from the cycad *Macrozamia* (Meeks et al. 2001; Campbell et al. 2008). *Nostoc* 29133 has previously been shown to adapt to a variety of stresses, including UVA radiation, herbicide methyl viologen, high light, and heavy metals (Soule et al. 2009; Moirangthem et al. 2014; Moparthy et al. 2016; Hudek et al. 2017; Li et al. 2018). This cyanobacterium is also considered a potential candidate for production of biofuels and many other high-value compounds (Moraes et al. 2017). The findings presented in this study show that *Nostoc* 29133 is relatively more tolerant to H_2O_2 than *Anabaena* 7120 due to its higher intrinsic constitutive H_2O_2 decomposition activity. This suggests that differences in H_2O_2 tolerance may exist even between closely related filamentous cyanobacteria. Further, the unique H_2O_2 stress-tolerant property of *Nostoc* 29133 is likely to add to its biotechnological value.

Materials and methods

Cyanobacterial strains and culture conditions

The batch cultures of *Nostoc* 29133 (*Nostoc punctiforme* strain ATCC 29133-S, also known as UCD 153, Campbell et al. 2008) and *Anabaena* 7120 (*Anabaena* sp. PCC strain 7120) were grown at 25 °C in BG11 liquid medium, pH 7.5, containing 17.6 mM sodium nitrate buffered with equimolar 4-(2-Hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) from stock cultures maintained on slants containing BG11 solid medium with 1.5% agar (Rippka et al. 1979). The cultures were continuously illuminated with cool fluorescent light (photon fluence rate of 20–23 µmol m^{-2} s^{-1}) during growth period, as described earlier (Moirangthem et al. 2014).

Determination of H_2O_2 tolerance

For H_2O_2 tolerance assay, the actively growing cultures, 100 ml each of *Nostoc* 29133 and *Anabaena* 7120 were harvested by centrifugation at 2300×*g* for 5 min and washed twice with fresh BG11 medium. Such cultures were inoculated at equal chlorophyll *a* (Chl *a*) concentration in flasks containing fresh BG11 medium (30 ml). The H_2O_2 stock (30% v/v; Merck, India) was diluted to 10 mM concentration with sterile Milli-Q water and subsequently different volumes of H_2O_2 were added to 30 ml cyanobacterial cultures at the start of the experiment to obtain concentrations ranging from 0 to 0.5 mM H_2O_2, and incubated as described

(Moirangthem et al. 2014). Growth was monitored periodically by measuring the concentration of Chl *a* spectrophotometrically (Cary 60, Agilent, USA) in methanolic extracts of cyanobacterial cultures using absorbance value at 663 nm × 13.43, where 13.43 represents extinction coefficient of Chl *a* (Mackinney 1941; Moirangthem et al. 2014).

Pulse amplitude modulated (PAM) fluorometry

The maximal efficiency of PSII photochemistry (F_v/F_m), which is a common and quick indicator of photosynthetic performance of cells, was measured in H_2O_2 treated and untreated cultures of both the cyanobacterial species after 15 min of dark adaptation using a Dual-PAM-100 fluorometer (Waltz, Effeltrich, Germany), as described earlier (Moirangthem et al. 2014). The F_v/F_m values were derived from $F_v = (F_m - F_0)$, where F_v represents the variable fluorescence signal, F_0 minimal fluorescence signal of dark adapted cells, and F_m the maximal fluorescence signal after application of a saturating light pulse (Schreiber et al. 1995).

Determination of total intracellular hydroperoxide

The total hydroperoxide levels were measured in cyanobacterial cells after incubation in the presence of different concentrations of H_2O_2 (0–0.5 mM) for one day by a ferrous oxidation/xylenol orange (FOX) assay method. H_2O_2 treated or untreated cultures were harvested by centrifugation at 2300×*g* for 5 min and washed twice with sterile Milli-Q water. A 0.8 ml methanolic solution containing 0.01% butylated hydroxytoluene (BHT) was then added to the cell pellets equivalent to 1.5 µg ml^{-1} Chl *a*. To this, 0.1 ml of reagent A prepared by mixing 2.5 mM ammonium ferrous sulfate in 0.25 mM sulfuric acid was added followed by addition of 0.1 ml of reagent B prepared by mixing 40 mM BHT and 1.25 mM xylenol orange in methanol. After incubation for 30 min at room temperature, this reaction mixture was centrifuged at 2300×*g* for 5 min to remove any cell debris. The supernatant fraction was used to measure the absorbance at 560 nm and the concentration of hydroperoxides was determined using the extinction coefficient ($\varepsilon_{560} = 4.3 \times 10^4$ M^{-1} cm^{-1}), as described earlier (DeLong et al. 2002; Moirangthem et al. 2014; Wolff 1994).

Determination of catalase activity

The cultures of *Nostoc* 29133 and *Anabaena* 7120 were exposed to 0 and 0.25 mM H_2O_2 for a day. The cells were centrifuged at 2300×*g* for 5 min, and the cell pellets obtained were washed twice with 36 mM potassium phosphate buffer (pH 7.4). The cell-free extracts were obtained by sonication of the cell pellets in the same buffer added with 1 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF), as described earlier (Moirangthem et al. 2014). The total protein in cell extracts was measured according to Bradford (1976). 50 µg of total protein was added to 50 mM phosphate buffer (pH 7) and 10 mM H_2O_2 in a final volume of 1 ml. Catalase activity was determined by measuring the disappearance of H_2O_2 at 240 nm using an extinction coefficient of 43.6 M^{-1} cm^{-1} (Beers and Sizer 1952).

Statistical analysis

All experiments were performed at least three times and the results are presented as mean ± standard deviation. The significant differences (*P* values less than 0.05) were analyzed by Student's two-sample *t* test (Microsoft word version 10) between control and H_2O_2 treated cyanobacterial cultures.

Results and discussion

Nostoc 29133 exhibits higher tolerance to H_2O_2 than *Anabaena* 7120

Cyanobacteria are often exposed to H_2O_2 generated in surrounding environments (Diaz and Plummer 2018; Zinser 2018). Being a small and neutral molecule, extracellular H_2O_2 can readily cross cell membranes and enter into cells. At high concentrations, H_2O_2 can be highly damaging to cellular growth and metabolism, especially if there is free ferrous iron available in the cell since $HO^{\bullet}$ radicals will be formed by Fenton chemistry (Latifi et al. 2009; Banerjee et al. 2013; Imlay 2013). In order to reveal the potential differences in H_2O_2 tolerance of filamentous *Nostoc* 29133 and *Anabaena* 7120, the active cultures of both cyanobacterial strains were subjected to increasing concentrations of exogenously added H_2O_2 (0.1, 0.25, and 0.5 mM), and Chl *a* content of cultures was measured for 6 days. This range of H_2O_2 concentration was chosen based on studies in *Aphanizomenon ovalisporum*, a filamentous cyanobacterium, which fails to survive in the presence of 0.5 mM H_2O_2 (Kaplan-Levy et al. 2015). When incubated with 0.1 mM H_2O_2, Chl *a* content of *Nostoc* 29133 (Fig. 1a) and *Anabaena* 7120 (Fig. 1b) decreased more or less to similar extent compared to their respective controls. However, a distinct difference between the two strains was observed with increased doses of H_2O_2. Whereas 0.25 and 0.5 mM H_2O_2 treatments resulted in a decrease in Chl *a* content of *Nostoc* 29133 by 12 and 20%, respectively, compared with untreated control, similar treatments led to complete loss of Chl *a* in *Anabaena* 7120. These results suggest that tolerance to H_2O_2 is higher in *Nostoc* 29133 compared to in *Anabaena* 7120, despite the two being taxonomically closely related to each other (order *Nostocales*) (Rippka et al. 1979). Differences in H_2O_2 tolerance among cyanobacteria have been described earlier, but such

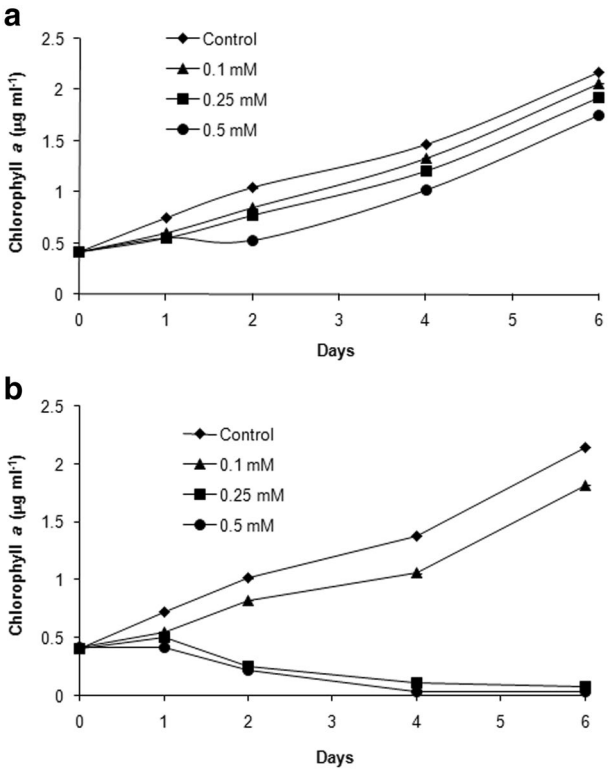

Fig. 1 Growth response of *Nostoc* 29133 and *Anabaena* 7120 to H_2O_2. The actively growing *Nostoc* 29133 (**a**) and *Anabaena* 7120 (**b**) cultures equivalent to 0.5 µg ml^{-1} of Chl *a* were seeded in fresh BG11 medium containing H_2O_2 (0–0.5 mM). The Chl *a* content of cultures was measured at periodic intervals for 6 days. The bars indicate standard deviation of three independent experiments and are smaller than the symbols

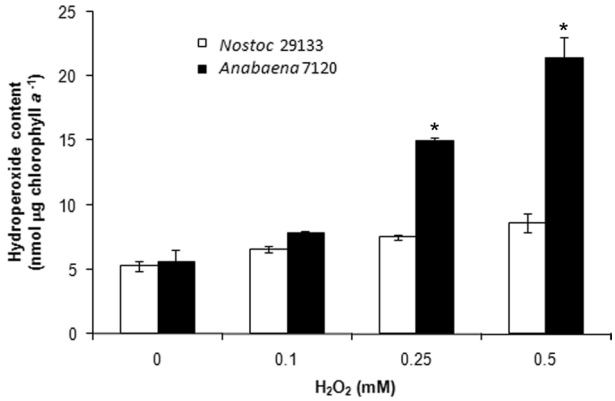

Fig. 2 Effect of H_2O_2 on total hydroperoxide level in *Nostoc* 29133 and *Anabaena* 7120. The cyanobacterial cultures treated or untreated with H_2O_2 for 1 day in fresh BG11 medium were used to measure the total intracellular hydroperoxide level by ferrous oxidation–xylenol orange method. The bars indicate standard deviation of three independent experiments. Asterisk (*$P<0.05$) on the bars represents significant difference as compared to respective control value

comparisons have been mostly between different taxonomical groups, for example, comparisons of *Anabaena* 7120 and *Synechocystis* sp. PCC 6803 (Pascual et al. 2010), and of *Cylindrospermopsis* and *Planktothrix* (Yang et al. 2018). Therefore, H_2O_2 tolerance seems to be a species-dependent feature in cyanobacteria, and may be related to differential accumulation of H_2O_2 and/or $HO^{\bullet}$ (Drábková et al. 2007a, b; Yang et al. 2018). This possibility was investigated by measuring the intracellular hydroperoxide concentrations in *Nostoc* 29133 and *Anabaena* 7120.

Nostoc 29133 displays lower intracellular hydroperoxide levels than *Anabaena* 7120

Total intracellular hydroperoxide levels (includes lipid hydroperoxides) were determined in the cultures of *Nostoc* 29133 and *Anabaena* 7120 exposed to H_2O_2 for 1 day by FOX assay (Wolff 1994; DeLong et al. 2002). As shown in Fig. 2, incubation with 0.1 mM H_2O_2 barely affected the hydroperoxide levels in either strain relative to their respective controls. However, exposure to higher concentrations of H_2O_2 led to differential increase in hydroperoxide levels; *Nostoc* 29133 displayed considerably lower levels relative to *Anabaena* 7120. It is highly likely that relatively lower accumulation of hydroperoxides within cells of *Nostoc* 29133 protected it from H_2O_2 lethality, as opposed to that in *Anabaena* 7120. To investigate if the ability to keep a low intracellular hydroperoxide concentration is also shown in a lower damage to the metabolism, the inhibition of PSII activity was measured in *Nostoc* 29133 and *Anabaena* 7120.

Nostoc 29133 exhibits lower inhibition of PSII performance than *Anabaena* 7120

PSII is a major target of H_2O_2 in many cyanobacteria (Drábková et al. 2007a, b; Nishiyama et al. 2011; Mikula et al. 2012; Yang et al. 2018; Piel et al. 2019). The maximum photochemical efficiency of PSII (F_v/F_m), an indicator of PSII electron transport capacity, is a quick and sensitive parameter to assess PSII performance in cyanobacteria (Schreiber et al. 1995). Thus, F_v/F_m was measured in whole cells of *Nostoc* 29133 and *Anabaena* 7120 following 1-day treatment with increasing concentrations of H_2O_2 (Fig. 3). The H_2O_2 treated cultures (0.1 and 0.25 mM) of *Nostoc* 29133 did not show any appreciable change in F_v/F_m values compared to control cultures, though F_v/F_m was inhibited to a smaller extent in 0.5 mM treated cultures. In *Anabaena* 7120, treatment with 0.1 mM H_2O_2 resulted in a mild inhibition of F_v/F_m compared with control; however, higher concentrations completely inhibited F_v/F_m. These results suggest lower inhibition of PSII performance in *Nostoc* 29133 than in *Anabaena* 7120, which may be an effect of tighter

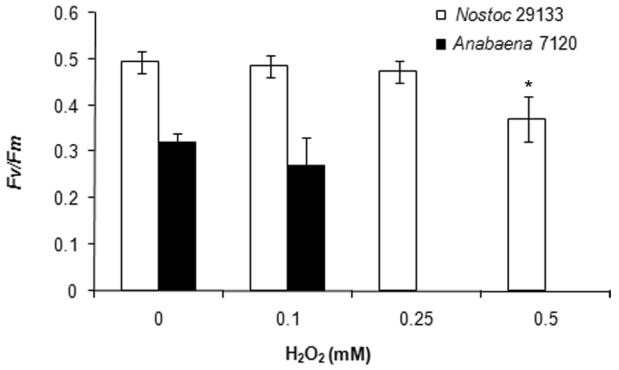

Fig. 3 Effect of H_2O_2 on maximum photochemical efficiency (F_v/F_m) of PSII in *Nostoc* 29133 and *Anabaena* 7120. The cyanobacterial cultures untreated or treated with different concentrations of H_2O_2 for 1 day in BG11 medium were harvested, washed, and resuspended in the same medium. Such cultures (Chl *a* concentration 10 µg ml^{-1}) were dark adapted for 15 min followed by F_v/F_m measurement. The bars indicate standard deviation of three independent experiments. Asterisk (*$P<0.05$) on the bar represents significant difference as compared to respective control value

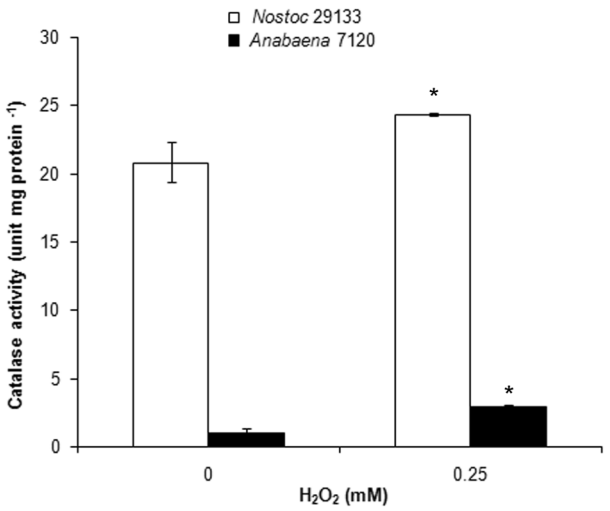

Fig. 4 Catalase activity assay in *Nostoc* 29133 and *Anabaena* 7120. The cyanobacterial cultures treated or untreated with H_2O_2 for 1 day in fresh BG11 medium were harvested, washed, and resuspended in 36 mM phosphate buffer (pH 7.4) and broken by ultrasonication. The cell extracts equivalent to 50 µg total protein was used for measuring catalase activity. The bars indicate standard deviation of three independent experiments. Asterisk (*$P<0.05$) on the bars represents significant difference as compared to respective control value

regulation of intracellular hydroperoxide levels in the former, thus limiting free H_2O_2 to inhibit the PSII.

Nostoc 29133 possesses higher catalase activity than *Anabaena* 7120

To probe the underlying reason for differential accumulation of hydroperoxides in *Nostoc* 29133 and *Anabaena* 7120, catalase activity was evaluated in the cell extracts of 1-day-old H_2O_2 treated and untreated cultures. The antioxidative enzymes, such as catalases and Prxs, are known to participate in scavenging H_2O_2 in cyanobacteria (Tichy and Vermaas 1999; Perelman et al. 2003; Bernroitner et al. 2009; Latifi et al. 2009; Banerjee et al. 2013). However, unlike catalases, Prxs are susceptible to H_2O_2-mediated overoxidation and inactivation (Pascual et al. 2010). As shown in Fig. 4, catalase activity was nearly 20-fold higher in control cultures of *Nostoc* 29133 than in control cultures of *Anabaena* 7120. While a 1.2-fold increase in catalase activity was observed in *Nostoc* 29133 after 0.25 mM H_2O_2 treatment, an approximately threefold increase in this activity was observed in *Anabaena* 7120, compared to their non-treated respective controls. However, such an increase in catalase activity was clearly not enough to prevent death by H_2O_2 in *Anabaena* 7120. As opposed to *Anabaena* 7120, a high intrinsic constitutive catalase activity seems to contribute to prompt and efficient decomposition of H_2O_2 resulting in lower intracellular hydroperoxide levels and higher tolerance to H_2O_2 in *Nostoc* 29133. This correlation of H_2O_2 tolerance and intrinsic catalase activity has also been demonstrated earlier in a comparative study between *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 (Gupta and Ballal 2015). It should be stressed that several cyanobacteria, including the filamentous forms, lack catalase activity and are sensitive to H_2O_2 (Bagchi et al. 1991; Bernroitner et al. 2009; Morris et al. 2011; Banerjee et al. 2012; Mikula et al. 2012; Yang et al. 2018; Piel et al. 2019). In this context, *Nostoc* 29133 may be unique, as it possesses catalase activity and a superior ability to tolerate H_2O_2. The findings presented here should pave the way for further in-depth characterization of catalases in this filamentous cyanobacterium and their roles in adaptation to H_2O_2 and other abiotic stress conditions, which presumably generate oxidative stress.

Conclusion

This study highlights differences in H_2O_2 tolerance between two closely related filamentous cyanobacteria *Nostoc* 29133 and *Anabaena* 7120. *Nostoc* 29133 exhibited lower reduction of chlorophyll *a* and PSII performance, as compared to *Anabaena* 7120 in response to exogenous H_2O_2. The higher tolerance of *Nostoc* 29133 to H_2O_2 was accompanied by a tighter control of intracellular hydroperoxide level supported by higher intrinsic constitutive catalase activity, in contrast to that in *Anabaena* 7120. H_2O_2 stress-tolerant photoautotroph, like *Nostoc* 29133, is likely to be an important biotechnological resource, and may be exploited as a potential source of valuable antioxidant catalase.

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Author contributions JB and LS conceived and designed the study. LS performed all the experiments. JB and LS interpreted the results. All the authors contributed to the writing of this manuscript. The final version of the manuscript was read and approved by all the authors.

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Conflict of interest The authors state that there is no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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APPROVAL OF RESEARCH PROPOSOAL

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ABSTRACT

STUDIES ON PHYSIOLOGICAL AND BIOCHEMICAL RESPONSE OF *NOSTOC PUNCTIFORME* ATCC 29133 AND *ANABAENA* SP. PCC 7120 TO OXIDATIVE STRESS INDUCED BY PEROXIDES

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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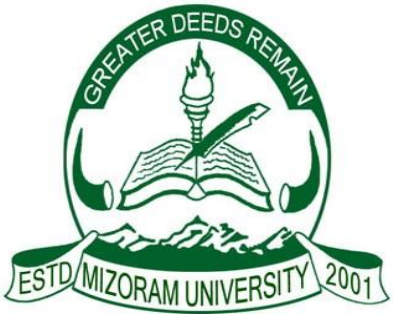

DEPARTMENT OF BIOTECHNOLOGY

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Abstract

Being photoautotrophic in nature, cyanobacteria must confront a variety of reactive oxygen species (ROS) generated from photosynthesis and/ or respiratory electron transport pathways. Sub-optimal growth conditions often encountered in natural environments increase the production of ROS in cyanobacteria. Excess ROS is highly detrimental to growth and survival of these ecologically, agriculturally, and biotechnologically important microorganisms. The present study embodies characterization of the physiological and biochemical responses of filamentous diazotrophic cyanobacteria *Nostoc punctiforme* strain ATCC 29133-S (*Nostoc* 29133) and *Anabaena* sp. PCC 7120 (*Anabaena* 7120) to oxidative stress induced by exogenous application of inorganic (H_2O_2) and organic hydroperoxides (*tert*-butyl hydroperoxide; *t*-BOOH), and methyl viologen (MV), a redox-cycling ROS-generating herbicide. The findings of this study are summarized below:

1. H_2O_2 tolerance ability of *Nostoc* 29133 was evaluated and compared with respect to *Anabaena* 7120. The results demonstrated that *Nostoc* 29133 was better able to tolerate H_2O_2-induced inhibition of chlorophyll *a* and photosystem II performance, as compared to *Anabaena* 7120. The intracellular hydroperoxide level (indicator of oxidative status) also did not exhibit as much a rise in *Nostoc* 29133, as it did in *Anabaena* 7120 after H_2O_2 treatment. Accordingly, *Nostoc* 29133 showed higher intrinsic constitutive catalase activity than *Anabaena* 7120 indicating that the superior tolerance of *Nostoc* 29133 stems from its higher ability to decompose H_2O_2, suggesting that even closely related filamentous cyanobacteria may differ in terms of H_2O_2 tolerance. H_2O_2 stress tolerant photoautotrophic cyanobacterium like *Nostoc* 29133 is likely to be an important biotechnological resource, and may be exploited as a potential source of valuable antioxidant catalase.

2. To determine if *Nostoc* 29133 and *Anabaena* 7120 also vary in their ability to tolerate organic hydroperoxide, the growth response of these two cyanobacteria to *t*-BOOH, an

analog of organic hydroperoxide, was examined. The results indicated that *Nostoc* 29133 and *Anabaena* 7120 are equally sensitive to *t*-BOOH, unlike H_2O_2. Further characterization of *Nostoc* 29133 revealed that increased accumulation of intracellular hydroperoxides resulting from inability of this cyanobacterium to quickly and completely detoxify *t*-BOOH, presumably via activity of peroxiredoxins, was the basis for its sensitivity to *t*-BOOH. The activity of antioxidative enzyme superoxide dismutase was not influenced by *t*-BOOH, but a new catalase isoenzyme was induced in response to this oxidant in *Nostoc* 29133. These findings suggest that in addition to *t*-BOOH detoxifying peroxiredoxins, catalase activity may also be important to tackle *t*-BOOH induced oxidative stress in *Nostoc* 29133. Further studies using knockout mutants may be necessary to determine the precise roles of each of these enzymatic antioxidants in mitigating organic hydroperoxide stress in cyanobacteria.

3. An investigation on the responses of *Anabaena* 7120 and *Nostoc* 29133 to MV (an endogenous producer of peroxides) revealed that these cyanobacteria can tolerate MV stress better when grown in the presence of nitrate (a fixed-nitrogen nutrient) than in its absence (diazotrophic growth). The MV-stressed nitrate grown *Anabaena* 7120 generated lower amount of hydroperoxides, as compared to when it is grown diazotrophically. This reduction in hydroperoxide level was independent of catalase activity. It was concluded that the protective effect of nitrate may be linked to its assimilation mechanism (nitrate and nitrite reduction), which consumes photosynthetically generated electrons (eight per nitrogen atom fixed), and thereby reduce electron flow to MV leading to reduced production of ROS and lower oxidative stress. Further studies using nitrate-assimilation defective mutants may provide stronger evidence for the role of nitrate in modulating MV tolerance in filamentous cyanobacteria. This may have implications for growth and survival of these important microorganisms in agricultural fields, which are often loaded with MV and nitrate fertilizers.